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Circulating Androgens and the Response to Stress

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADX	adrenalectomized
AR	androgen receptor
AVP	arginine vasopressin
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CORT	corticosterone
CNS	central nervous system
CRF	corticotropin-releasing factor
CRH	corticotropin-releasing hormone
DHT	dihydrotestosterone
E	estrogen
ER	estrogen recepter
GABA	gamma-aminobutyric acid
GDX	gonadectomized
GnRH	gonadotropin-releasing hormone
HPA	hypothalamo-pituitary-adrenal
HPG	hypothalamo-pituitary-gonadal

HRE	hormone response element
hsp	heat shock protein
INT	intact
IR	immunoreactive
ISHH	*in situ* hybridization histochemistry
MeA	medial amygdala
POMC	proopiomelanocortin
PVN	paraventricular nucleus of the hypothalamus
PVN_{dp}	dorsal parvocellular subdivision of PVN
PVN_{M}	magnocellular subdivisions of the PVN
PVN_{mpd}	dorsal medial parvocellular subdivision of PVN
PVN_{mpv}	ventral medial parvocellular subdivision of the PVN
PVN_{p}	parvocellular subdivisions of the PVN
RIA	radioimmunoassay
T	testosterone

CHAPTER I

INTRODUCTION

A stressful stimulus can be defined as a threat (real or perceived) to an organism's survival, integrity, or reproductive success. The adaptive biological reactions to such a stimulus have been termed the stress response. The animal's full attention and integrated action may be required in order to achieve survival and to eliminate the threatening stimulus. To this end, the stress response involves changes in several physiological and behavioral parameters. For example, activation of the endocrine and the autonomic nervous systems serves to increase the probability of survival of the organism and to terminate the stress response once the source of the stress has been eliminated.

In mammals, the hypothalamo-pituitary-adrenal (HPA) axis is an important component of an organism's adaptive response to stress. Neurons involved in the initiation of this response synthesize corticotropin-releasing hormone (CRH). These neurons are mainly located in the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus. These neurons project to the median eminence where they secrete CRH into the hypophysial portal vasculature. CRH stimulates corticotrophs of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the peripheral circulation. ACTH then induces the secretion of corticosterone (CORT) from the adrenal cortex. During stressful events, corticosteroids act to

preserve the integrity of the organism by altering several physiological parameters. For example, CORT increases glucose mobilization in order to provide the animal with the necessary energy to contend with the stressor.

Gonadal hormones are also known to influence HPA activity. A sex difference in basal CORT titers and in the CORT response to stress has been demonstrated (Kitay, 1961). Females have higher basal CORT levels and enhanced CORT responses to stress compared to males. The greater secretion in females is believed to be the result of estrogen acting to enhance HPA activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992). Conversely, in males androgens may inhibit HPA activity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994; Viau and Meaney, 1991b). Previous studies have shown that prepuberal gonadectomy of the male rat and hamster leads to enhanced levels of CORT which correlate with increases in ACTH bioactivity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971). In gonadectomized male rats, the CORT and ACTH responses to foot shock or open field stressors are enhanced compared to intact animals (Handa *et al.*, 1994). Furthermore, treatment of gonadectomized rats with testosterone or the non-aromatizable androgen, dihydrotestosterone (DHT), normalizes post-stress plasma CORT and ACTH levels. These data provide further evidence that the sex difference in HPA activity arises as a result of the circulating gonadal steroid environment.

Several possible mechanisms could explain androgen's inhibition of HPA activity. Androgens could be inhibiting synthesis and/or secretion of hypothalamic

CRH (or other corticotropin-releasing factors such as arginine vasopressin, AVP). Alternatively, androgens may influence the negative feedback response of the HPA axis. However, this is not supported by evidence that gonadectomy of male rats does not alter the concentration or affinity of type I or type II CORT receptors in the hippocampus or hypothalamus (Handa *et al.*, 1994). Furthermore, anterior pituitary responsiveness to CRH is not altered following gonadectomy of male rats (Handa *et al.*, 1994). The possibility remains that androgens could be influencing hypothalamic CRH synthesis, storage, or secretion.

It is important to characterize the effect(s) of gonadal hormones on the stress response. Dysfunction of the stress response is believed to lead to adjustment disorders such as depression and panic disorder. These disorders occur much more often in females than in males (Weissman and Klerman, 1977; Katschnig and Amering, 1990). While it is clear that estrogen may play an important role in predisposing a female to such disorders, it is equally as important to determine what role androgens may play in protecting a male from dysfunction of the stress response. Thus, this dissertation tested the hypothesis that **in the male rat, circulating androgens modulate the neuronal circuitry responsible for mediating neuroendocrine and behavioral responses to stressful stimuli.** This hypothesis was tested in two parts. First, the effect of circulating gonadal androgens on the HPA axis was examined, and secondly, the effect of circulating androgens on other neuroendocrine and behavioral responses to stress was evaluated.

Four experiments were conducted to test this general hypothesis. The first

experiment tested the hypothesis that androgens affect the HPA axis by reducing hypothalamic CRH levels. Thus, the first experiment examined the effect of gonadectomy and the non-aromatizable androgen, DHT, on hypothalamic CRH content and CRH-immunoreactive (IR) cell numbers within the PVN of male rats. The second experiment was performed to test the hypothesis that the influence of androgens on CRH content and CRH immunoreactivity in the hypothalamus are a consequence of changes in CRH gene expression as determined by measuring steady state levels of CRH mRNA. CRH mRNA levels in the PVN of intact, gonadectomized, and gonadectomized, DHT-treated animals were evaluated using *in situ* hybridization histochemistry (ISHH). The third experiment tested the hypothesis that androgens regulate CRH by acting directly within CRH containing cells of the PVN. The regulation of cellular processes by androgens requires the presence of the androgen receptor (AR) which acts as a ligand-responsive transcription factor. In order for androgens to directly regulate hypothalamic CRH, AR must be expressed within CRH containing cells of the PVN. Therefore, this experiment tested for the presence of AR immunoreactivity within CRH neurons in the PVN of male rats. The purpose of the final study was to test the hypothesis that circulating androgens influence neuroendocrine, autonomic, and behavioral responses to a psychological stressor. The effects of conditioned stress were examined in male rats that were intact, gonadectomized, or gonadectomized and treated with DHT. The effects of gonadal androgens on the stress-induced increases in plasma corticosterone, ACTH, prolactin, renin concentration, and renin activity were examined. In addition, this

experiment examined the influence of androgens on the stress-induced changes in defecation and several behavioral parameters.

CHAPTER II

REVIEW OF RELATED LITERATURE

The "Stress Response"

The terms "stress" and "homeostasis" were coined to describe very basic yet significant concepts of interaction between an organism and its environment. The concept of "homeostasis" was described by Cannon (1939) as a closed system of processes which defend the steady state against environmental stimuli that tend to disturb the balance. Selye (1950) observed that the body reacts to any nonspecific noxious agent or tissue damage with the same syndrome: hypertrophy of the adrenal, atrophy of the thymus, and development of gastric ulcers. He thus introduced the word "stress" to describe the state of the organism during the response to the noxious or threatening stimuli. More recently, the term "stress" has been used to refer to any perturbations of homeostasis and may be physiological or psychological in nature.

In mammals, most physiological systems are affected in some way by stressful stimuli. The endocrine system, the autonomic system, and the immune system are all sensitive to such environmental changes. Stressful stimuli elicit increases in plasma levels of several hormones including corticosterone, prolactin, and oxytocin. Autonomic outputs evoke changes in the cardiovascular, respiratory, and other visceral systems. Changes in immune function include a suppression of T lymphocyte

mitogen responsiveness (Cunnick *et al.*, 1991). All of these changes serve to preserve the integrity of the organism by increasing the readiness and execution of behaviors. This will ultimately enhance the probability of survival of the organism and eliminate the source of the stress. In addition, these physiological reactions ensure the termination of the stress response once the source of the stress has been eliminated. Thus, the "stress response" is an integral part of an adaptive biological system.

The Hypothalamo-Pituitary-Adrenal Axis

The hypothalamo-pituitary-adrenal (HPA) axis is a major component of the stress response (see Figure 1). Parvocellular neurosecretory neurons located in the paraventricular nucleus of the hypothalamus (PVN) synthesize a neuropeptide hormone, corticotropin-releasing hormone (CRH). These neurons project to the median eminence and terminate around the primary capillaries of the hypophysial portal circulation. The hypothalamus secretes CRH (and other corticotropin-releasing factors) into the hypophysial portal vasculature (Plotsky and Vale, 1984). Corticotropin-releasing hormone is the primary stimulus causing corticotrophs of the anterior pituitary to synthesize and secrete adrenocorticotropic hormone (ACTH) into the peripheral circulation (Rivier *et al.*, 1982). Subsequently, ACTH stimulates the cells of the zona fasciculata to synthesize and secrete glucocorticoids (predominantly corticosterone, CORT, in rats and cortisol in humans; Makara, 1985; Baxter 1986).

The activity of the HPA axis is influenced by both a circadian pacemaker and

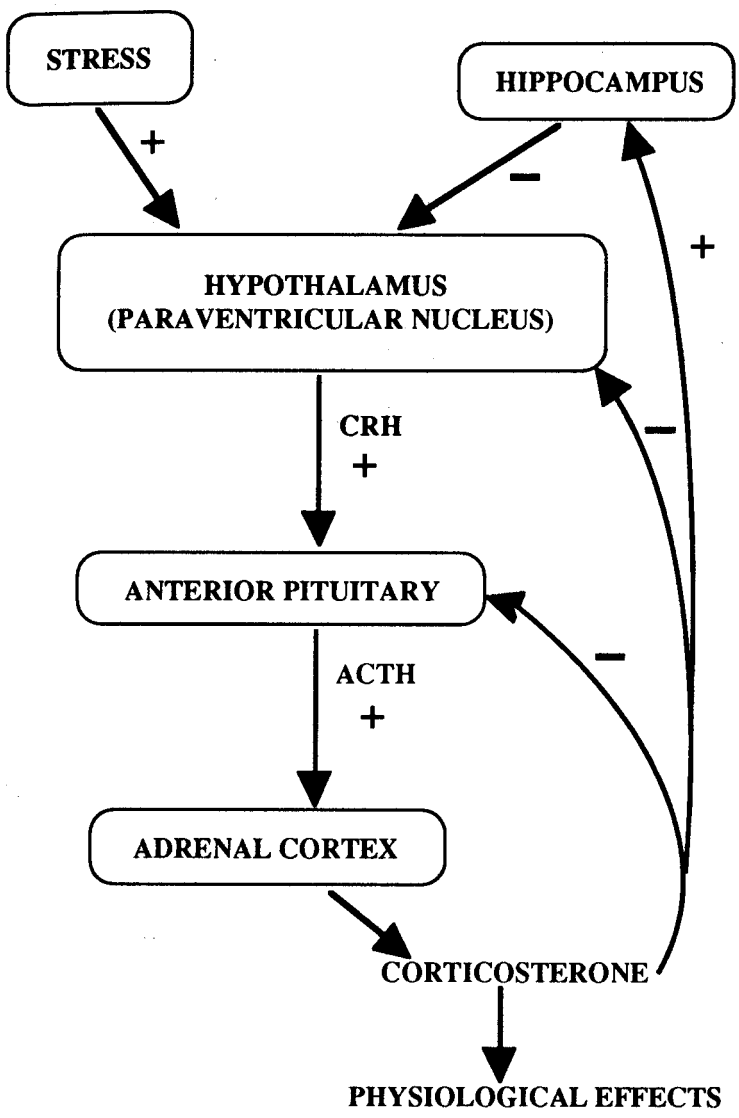


Figure 1. The hypothalamo-pituitary-adrenal (HPA) axis. In response to stress, corticotropin-releasing hormone (CRH) is secreted from the hypothalamus. CRH stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) which subsequently stimulates the release of corticosterone (CORT) from the adrenal cortex. In addition to its systemic effects, CORT feeds back on the hippocampus, hypothalamus, and pituitary to bring CRH, ACTH, and CORT secretion back to baseline levels.

environmental stimuli. Plasma CORT levels exhibit a diurnal rhythm with peak circulating CORT titers at the onset of the animal's active period (Dallman *et al.*, 1987). In addition to the circadian fluctuations, HPA activity increases rapidly in response to stressful stimuli. The HPA response to environmental perturbation corresponds with the circadian rhythm of the axis in that the response is greatest in the morning when plasma CORT titers are at their circadial nadir (Bradbury *et al.*, 1991). During stress, corticosteroids feedback on the hippocampus, hypothalamus, and pituitary to bring CRH, ACTH, and adrenal steroid secretion back to basal levels (Kim and Kim, 1961; Jingami *et al.*, 1985; Sapolsky *et al.*,1990).

The role of circulating CORT during stress is believed to be protective in nature. Corticosteroids dampen physiological responses involving immunity, inflammation, water resorption, and glucose utilization (for rev. see Munck *et al.*, 1984; Axelrod and Reisine, 1984). The exposure to a noxious or threatening stimulus induces defense reactions such as inflammation following tissue damage. These responses may become detrimental if they are not terminated once the threatening stimulus has disappeared. It has been suggested that during stress, glucocorticoids inhibit the synthesis and release of several intracellular mediators of these defense reactions (Munck *et al.*, 1984). Thus, corticosteroids act to prevent an overreaction to stress. In addition, glucocorticoids increase the synthesis of enzymes involved in gluconeogenesis in hepatocytes (Weber *et al.*, 1955; Weber *et al.*, 1964). This serves to increase the available energy such that the organism is better equipped to contend with or escape from a threatening stimulus.

In addition to CORT's effects on peripheral physiology, CORT also has profound effects upon the central nervous system. Adrenalectomy and corticosterone treatment have been shown to alter catecholaminergic, monoaminergic, peptidergic, and amino acid neurotransmitter systems (for rev. see McEwen *et al.*, 1986). For example, corticosterone treatment increases dopamine turnover and release (Versteeg et al., 1983; Imperato et al., 1989) and decreases hippocampal norepinepherine-stimulated cAMP production (Harrelson and McEwen, 1987). The effects of CORT on these neurotransmitter systems may be involved in the regulation of mood, perception, attention and learning (McEwen *et al.*, 1986).

Although glucocorticoids can serve a protective function, chronically high levels of circulating CORT can be detrimental. Chronically elevated plasma CORT can lead to steroid diabetes, hypertension, osteoporosis, immunosuppression, neuronal loss, impaired reproductive function, and disturbances in mood and cognitive function (Seyle and Tuchweber, 1976; Munck et al., 1984; Sapolsky et al., 1985; McEwen et al., 1986). Since these are all symptoms of aging, it has been suggested that chronically elevated CORT titers may be related to the aging process and age-related neuropathology (Landfield et al., 1978; Landfield et al., 1981; Sapolsky et al., 1986).

Corticotropin-Releasing Hormone (CRH) and Factors (CRFs)

Over a decade ago, Vale and colleagues (1981) sequenced and characterized CRH, the 41-amino acid peptide that serves as the major physiological stimulus for adenohypophysial ACTH secretion. The evidence indicating that CRH induces

adenohypophysial CRH secretion is extensive. CRH has been shown to stimulate the release of ACTH both *in vitro* (Vale *et al.*, 1983a) and *in vivo* (Rivier *et al.*, 1982). Stimulation of ACTH release by endogenous CRH is attenuated by anti-CRH antibodies (Rivier *et al.*, 1984a; Van Oers *et al.*, 1989). Also, administration of CRH antisera nearly abolishes the pituitary and adrenal responses to a variety of stressors (Rivier and Vale, 1983; Ono *et al.*, 1985). In addition to increasing secretion of ACTH, CRH increases the synthesis and release of other adenohypophysial products of the ACTH precursor peptide, proopiomelanocortin (POMC; Rivier and Plotsky, 1986; Vale *et al.*, 1983b) and the level of POMC mRNA (Bruhn *et al.*, 1984; Dallman *et al.*, 1985) in corticotroph cells of the anterior pituitary.

Several other hypothalamic substances including arginine vasopressin, (AVP; Gillies *et al.*, 1982; Rivier *et al.*, 1984b; Giguere and Labrie, 1982; Rivier and Vale 1983; Antoni *et al.*, 1983; Vale *et al.*, 1983a), oxytocin (Antoni *et al.*, 1983; Vale *et al.*, 1983a), and catecholamines (Vale and Rivier, 1977) synergize with CRH in its effects on adenohypophysial ACTH release. These substances have been proposed to be "CRFs". Interactions between these CRFs appear to be physiologically important since the secretion of each CRF is stimulus specific (Plotsky *et al.*, 1985a; Plotsky *et al.*, 1985b). For example, during insulin-induced hypoglycemia increases in plasma ACTH levels have been shown to accompany increases in AVP, but not CRH or catecholamines, in the portal blood (Plotsky *et al.*, 1985a).

Several laboratories have demonstrated that neurohypophysial AVP potentiates

the effect of CRH on pituitary corticotrophs in both rats (Giguere and Labrie, 1982; Rivier and Vale, 1985a; Graf *et al.*, 1985) and humans (Orth *et al.*, 1985). AVP acts through its receptor subtype, V1, (Rivier *et al.*, 1984b) to potentiate CRH-stimulated cAMP accumulation (Giguere and Labrie, 1982). However, it is currently thought that CRH is the only hypothalamic factor capable of inducing POMC gene expression (Levin *et al.*, 1989). AVP may potentiate CRH action by increasing CRH receptors on the corticotroph cell surface (Madsen *et al.*, 1991; Jia *et al.*, 1991). AVP may accomplish this through its action on a functionally distinct population of corticotrophs which does not contain CRH receptors (Schwartz and Vale, 1988; Jia *et al.*, 1991).

In the rat, oxytocin amplifies the ACTH response to CRH much in the same way as AVP does (Antoni *et al.*, 1983; Vale *et al.*, 1983a). The effects of oxytocin and AVP on ACTH secretion are not additive (Gibbs *et al.*, 1984). It is therefore likely that these neuropeptides act via the same intracellular pathways. Although oxytocin has not been shown to influence plasma ACTH levels in humans (Lewis and Sherman, 1985), oxytocin appears to be an important CRF in the rat. The levels of oxytocin in the hypophysial portal blood of the rat are 50-100 times higher than the concentration in peripheral blood (Gibbs, 1985; Horn *et al.*, 1985). The source of this hypophysial portal oxytocin is the axons of magnocellular neurons of the supraoptic and accessory nuclei which are passing through the median eminence (Antoni *et al.*, 1986).

The CRH Gene

The gene encoding the CRH prohormone has been cloned and sequenced in several species (Furutani *et al.*, 1983; Shibahara *et al.*, 1983; Jingami *et al.*, 1985; Thompson *et al.*, 1987). The genes encoding rat and human CRH precursors are highly homologous and are comprised of two exons separated by an intervening intron (see Figure 2; Jingami *et al.*, 1985; Thompson *et al.*, 1987). The nucleotide sequence encoding the entire CRH prohormone is located on the second exon while the first exon encodes most of the 5'-untranslated regions of the mRNA. Comparison of the human and rat gene reveals a high degree of homology not only in the peptide encoding region, but also in the 5'-flanking sequence. The 5'-flanking region is likely to contain DNA sequence elements responsible for tissue specific expression as well as second messenger and steroid regulation of gene expression. For example, a cAMP-responsive element has been localized to a 59-base pair region located between 238 and 180 base pairs 5' to the CRH cap site (Seasholtz *et al.*, 1988).

Induction of immediate early genes such as c-fos may be involved in CRH gene expression. Induction of c-Fos immunoreactivity has been observed in CRH-immunoreactive (IR) parvocellular neurons of the PVN following various stressors (Ceccatelli *et al.*, 1989a) as well as following adrenalectomy (Jacobson *et al.*, 1990). Recently, a temporal correlation was reported in which the enhanced expression of CRH mRNA in the PVN following immobilization stress was preceded by the induction of c-fos mRNA (Imaki *et al.*, 1992). However, this is contradicted by a study demonstrating that CRH heterogeneous nuclear RNA and c-fos mRNA rise

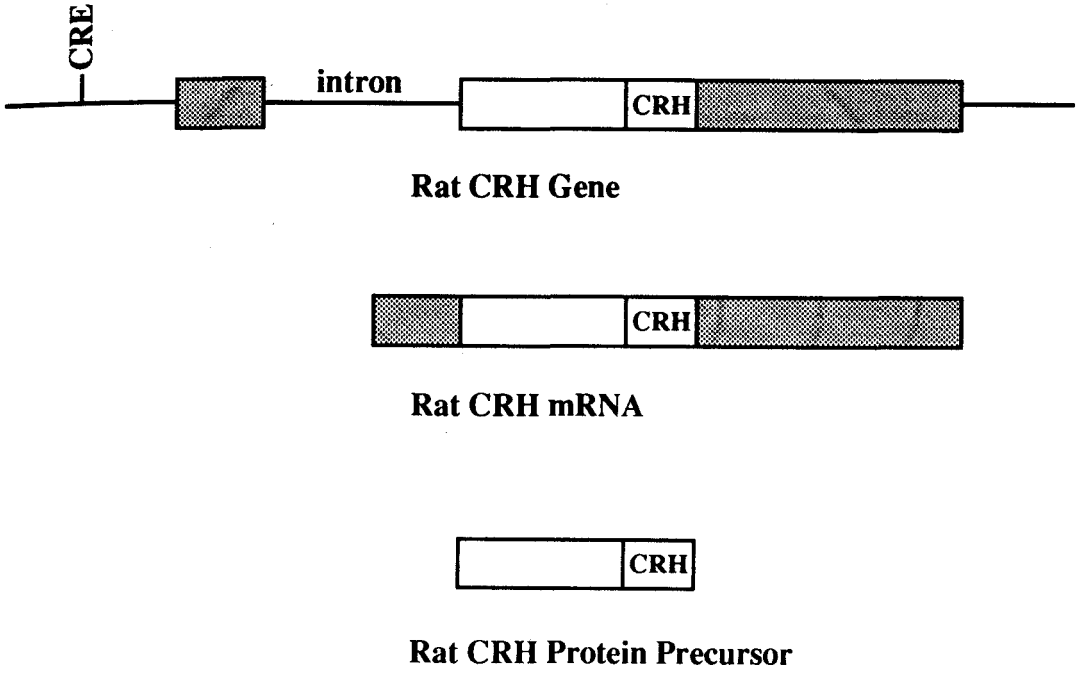


Figure 2. Structural organization of the rat corticotropin-releasing hormone (CRH) gene, mRNA (1400 nucleotides), and protein precursor (187 amino acids). The exons of the gene are shown as blocks. The intron, 5'- and 3'-flanking sequences are shown as lines. The 5'- and 3'-untranslated regions are shaded. The cAMP-responsive element (CRE) is indicated. The location of the CRH peptide is indicated by CRH (adapted from Thompson *et al.*, 1987).

concomitantly following blockade of corticosterone synthesis with metyrapone (Herman *et al.*, 1992).

Localization of CRH Neurons

The PVN is composed of a cluster of neurons that lie dorsally along the third ventricle (see Figure 3). The PVN contains at least three functionally and anatomically distinct groups of neurons (see Figure 4; for rev. see Swanson, 1987).

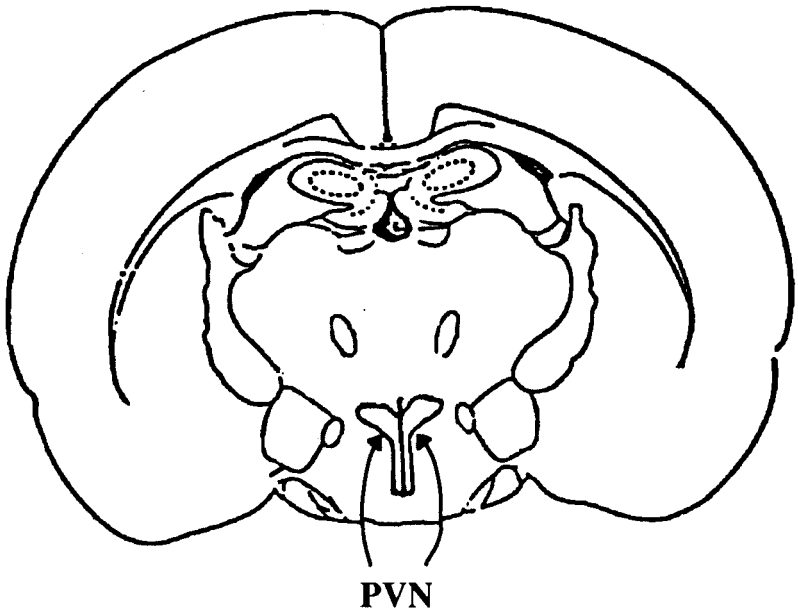


Figure 3. The paraventricular nucleus of the hypothalamus (Interaural 7.20 mm, Bregma -1.80 mm; adapted from Paxinos and Watson, 1986).

The parvocellular neurosecretory group consists of neurons that project to the neurohemal zone of the median eminence, where they release hormones into the hypophysial portal circulation. These cells are found in the dorsal medial parvocellular and periventricular subdivisions of the PVN. The magnocellular neurosecretory group consists of large neurons whose axons project to the posterior pituitary where they release vasopressin and oxytocin into the general circulation. These neurons are concentrated in the anterior, medial, and posterior magnocellular subdivisions of the PVN. The third group of neurons are localized to the dorsal, ventral medial and lateral parvocellular subdivisions of the PVN. These neurons give rise to descending projections to other areas of the hypothalamus, the brainstem and the spinal cord. These neurons are believed to influence the autonomic nervous

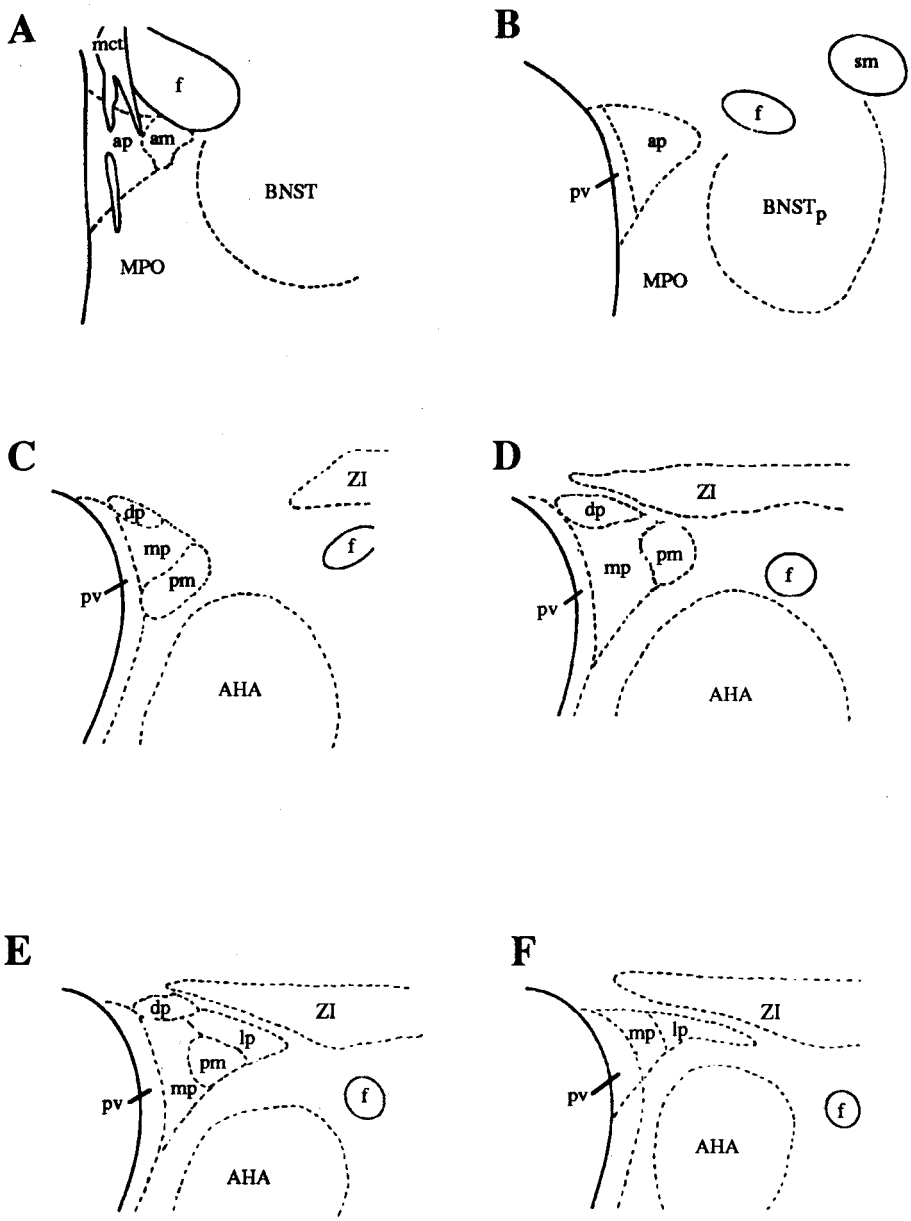


Figure 4. The subdivisions of the paraventricular nucleus of the hypothalamus (PVN). Frontal sections are arranged from rostral (A) to caudal (F). AHA, anterior hypothalamic area; am, anterior magnocellular part of the PVN; ap, anterior parvocellular part of the PVN; $BNST_p$, bed nucleus of the stria terminalis (preoptic part); dp, dorsal parvocellular part of the PVN; f, fornix; lp, lateral parvocellular part of the PVN; mct, medial cortico-hypothalamic tract; mp, medial parvocellular part of the PVN; pm, posterior magnocellular part of the PVN; pv, periventricular part of the PVN; ZI, zona incerta (adapted from Swanson, 1987).

system, the output of the central gray and reticular formation, and the central relay of visceral and nociceptive information.

CRH containing neurons are found in all 8 divisions of the PVN, however most are found in the dorsal medial parvocellular part (for rev. see Sawchenko and Swanson, 1990). The axons of these cells project laterally around the fornix and end in the neurohemal zone of the median eminence where CRH is released into the hypophysial portal blood.

CRH neurons are found in many other areas of the central nervous system. Other hypothalamic sites in which CRH-IR cells have been observed include the arcuate, periventricular, premammillary, preoptic, supraoptic, and suprachiasmatic nuclei (for rev. see Sawchenko and Swanson, 1990). The majority of the projection fields of these CRH-IR cells are unknown. Extrahypothalamic brain areas containing CRH neurons include a dense cluster of CRH neurons within the central nucleus of the amygdala (CeA) and in the bed nucleus of the stria terminalis (BNST; Swanson *et al.*, 1983; Moga and Gray, 1985; Gray and Magnuson, 1987). Although the function of these cells has not been fully characterized, these neurons may serve to coordinate endocrine, autonomic, and behavioral responses to stress (for rev. see Menzaghi *et al.*, 1993).

Localization of CRH mRNA coincides with immunocytochemical data of peptide localization. Using *in situ* hybridization histochemistry (ISHH), CRH mRNA was observed in both the parvocellular and magnocellular regions of the PVN as well as in the supraoptic nucleus of the hypothalamus (Young *et al.*, 1986a; Lightman and

Young, 1987). Additionally, Northern blot analysis has revealed CRH mRNA in RNA extracted from the PVN, amygdala, BNST, and supraoptic nucleus (Beyer *et al.*, 1988).

Other neuropeptides including AVP are localized within various subsets of the parvocellular PVN CRH neurons. Furthermore, adrenalectomy induces expression of AVP in the majority of CRH cells in the PVN (Roth, et al., 1982; Sawchenko, et al., 1984), and the ratio of AVP:CRH release from the median eminence *in vitro* increases from 2:1 in the intact rat to 8:1 in adrenalectomized animals (Holmes *et al.*, 1986). This could be important since AVP is believed to potentiate the effect of CRH on ACTH secretion. In addition, oxytocin (Sawchenko *et al.*, 1984), neurotensin (Ceccatelli *et al.*, 1989b), dynorphin (Roth *et al.*, 1983), enkephalin (Ceccatelli *et al.*, 1989b; Hokfelt *et al.*, 1983), and enkephalin mRNA (Pretel and Piekut, 1990) have been localized within CRH neurons in the PVN. The function of these colocalized peptides is unknown, but one peptide may influence the action of the other at the level of the anterior pituitary.

Neuroanatomical Inputs to the PVN

The neuroanatomical inputs to the PVN arise from both extrahypothalamic and intrahypothalamic sources (for rev. see Swanson and Sawchenko, 1983; Swanson, 1987; Palkovits, 1987). Extrahypothalamic inputs are derived from three general systems: the limbic system, the lower brain stem, and the circumventricular organs (see Figure 5). Limbic system structures which influence the PVN include the

hippocampus (subiculum), the amygdala, and the septum. Most of these structures do not send direct pathways to the PVN but rather relay through the septum, the bed nucleus of the stria terminalis, or various hypothalamic nuclei (Sawchenko and Swanson, 1983).

Lower brainstem inputs to the PVN originate from the raphe nuclei, the locus coeruleus, the parabrachial nucleus, the nucleus of the solitary tract, and the reticular formation. Cells in the lateral reticular nucleus (A1-C1 cells in the ventrolateral medulla), the medial part of the nucleus of the solitary tract (A2-C2 cells in the dorsomedial medulla), and the locus coeruleus (A6 cell group) give rise to the majority of the noradrenergic and adrenergic innervation of the PVN (Palkovits *et al.*, 1980a; Palkovits *et al.*, 1980b; Sawchenko and Swanson, 1982; Kiss *et al.*, 1983). The axons of these cells ascend via a periventricular route or via the ventral tegmental bundle-medial forebrain bundle system or to the PVN. Serotoninergic innervation of the PVN originates from the median and dorsal raphe nuclei (Sawchenko *et al.*, 1983; Palkovits *et al.*, 1977).

Circumventricular organ innervation of the PVN arises from the subfornical organ and the organum vasculosum lamina terminalis. The pathway from the subfornical organ to the PVN contains angiotensin II immunoreactivity (Lind *et al.*, 1984) and is activated by hemorrhage (Abdelaal *et al.*, 1974; Mangiapani and Simpson, 1980; Knepel *et al.*, 1982; Lind *et al.*, 1983). Thus, subfornical organ innervation of the PVN is likely to be involved in the release of CRH, vasopressin, and oxytocin during hypovolemia (Swanson, 1987).

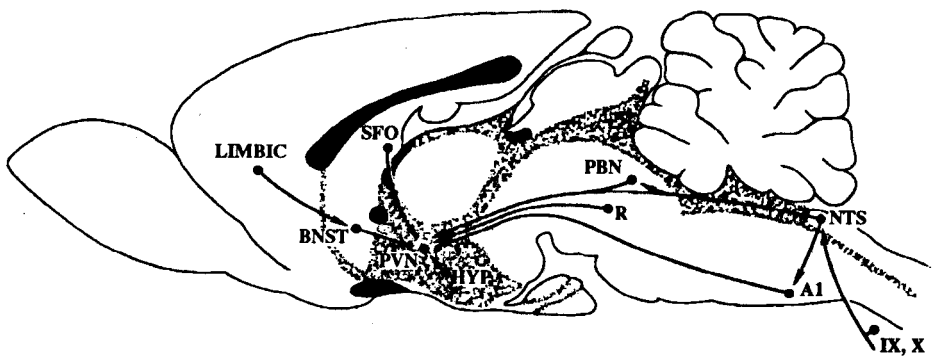


Figure 5. Summary of major neural inputs to the paraventricular nucleus of the hypothalamus (PVN). Limbic system inputs include projections from the hippocampus and amygdala. A1, A1-C1 cells in the ventrolateral medulla; BNST, bed nucleus of the stria terminalis; HYP, various hypothalamic nuclei; IX, glossopharyngeal nerve; NTS, nucleus of the solitary tract; PBN, parabrachial nucleus; R, raphe nuclei; SEP, septum; SFO, subfornical organ; X, vagus nerve (adapted from Swanson, 1987).

Intrahypothalamic inputs to the PVN arise from almost all of the hypothalamic and preoptic nuclei (Swanson and Cowan, 1975; Swanson, 1976; Saper *et al.*, 1976; Saper *et al.*, 1978; Saper *et al.*, 1979; Silverman *et al.*, 1981). Currently, most of this innervation has not been neurochemically characterized. Those inputs that have been characterized are peptidergic, using transmitters such as angiotensin II and neuropeptide Y (Bai *et al.*, 1985). Using electron microscopic immunocytochemistry, synaptic specializations between magnocellular neural processes and CRH-IR perikarya have been observed (Leranth *et al.*, 1983). Thus, it is likely that magnocellular neurons within the PVN (probably containing vasopressin or oxytocin)

also innervate CRH neurons.

Signals indicating the existence of physical and psychological stress reach the PVN via multiple neural pathways. Anatomical and physiological evidence suggest that somatosensory and viscerosensory information reaches the PVN via multi-synaptic neural pathways (Palkovits, 1987; Swanson, 1987). This information reaches the medullary and pontine reticular formation and biogenic amine containing cells groups. Projections from these cells ascend to the PVN via the ventral tegmental tract and medial forebrain bundle. For example, the nucleus of the solitary tract receives visceral information from the vagus and glossopharyngeal cranial nerves. These signals are then conveyed to the PVN. This example is probably the most direct route to the PVN for viscerosensory information. In contrast, psychogenic stress probably requires neural processing in the cerebral cortex (Swanson, 1987). This cognitive information is then carried to limbic regions such as the hippocampus and amygdala. The hippocampus and amygdala then relay the signals to the PVN. Although direct innervation of the PVN from the amygdala has been demonstrated (Gray *et al.*, 1989) both the hippocampus and amygdala send indirect connections to the PVN via the septum and bed nucleus of the stria terminalis (Swanson, 1987; Cullinan *et al.*, 1993).

Neurotransmitters Influencing CRH Secretion

Several neurotransmitter systems are believed to influence the secretion of CRH from parvocellular PVN neurons (for rev. see Tsagarakis and Grossman, 1990;

Table 1. Neurotransmitters that control hypothalamic CRH release.

STIMULATORY	INHIBITORY
Acetylcholine Norepinepherine Serotonin Angiotensin II Neuropeptide Y Activin	GABA Opioids Substance P Atrial Natriuretic Peptide Vasopressin

Whitnall, 1993). In general, acetylcholine, catecholamines, serotonin, and some neuropeptides such as angiotensin II and neuropeptide Y are believed to have a stimulatory effect on hypothalamic CRH secretion. In contrast, gamma-aminobutyric acid (GABA), endogenous opioids, and neuropeptides such as substance P are thought to inhibit secretion of CRH into the hypophysial portal blood. These neurotransmitter effects on hypothalamic CRH release are summarized in Table 1.

A great deal of evidence supports a stimulatory effect of acetylcholine on ACTH secretion. Intracerebroventricular injection or median eminence implantation of the cholinergic agonist, carbachol, yielded an increase in plasma CORT levels (Krieger and Krieger, 1970; Abe and Hiroshige, 1974). Furthermore, implantation of the cholinergic antagonist atropine rostral to the PVN was found to decrease the ACTH responses to surgical and ether stress (Hedge and Smelik, 1968; Hedge and De Wied, 1971). *In vitro* studies using hypothalamic explants indicated that

administration of acetylcholine increased CRH bioactivity and CRH as measured by radioimmunoassay (Hillhouse *et al.*, 1975; Buckingham and Hodges, 1979; Suda *et al.*, 1987; Tsagarakis *et al.*, 1988; Calogero *et al.*, 1988a). *In vivo*, administration of acetylcholine resulted in an increase in hypophysial portal CRH in anesthetized rats (Plotsky *et al.*, 1987). Both nicotinic and muscarinic receptors may be involved in this response although the muscarinic response is probably the dominant one (Tsagarakis *et al.*, 1988). This is consistent with the distribution of cholinergic receptors as the muscarinic receptor is more predominant in the hypothalamus (Dolly and Barnard, 1984).

The effect of catecholamines on hypothalamic CRH secretion is somewhat controversial (for rev. see Plotsky *et al.*, 1989; Whitnall, 1993). However, the strongest evidence indicates that catecholamines stimulate HPA activity. Administration of norepinepherine to rats under basal conditions increases circulating ACTH (Szafarcayk *et al.*, 1987; Plotsky *et al.*, 1987; Guillaume *et al.*, 1987). Electrical or chemical activation of catecholaminergic afferent pathways to the hypothalamus yields increases in CRH, ACTH, and CORT secretion while blockade or destruction of these pathways decreases HPA activity (Plotsky *et al.*, 1989). In vitro, norepinepherine stimulation of CRH secretion is blocked by β-adrenergic antagonists (Tsagarakis *et al.*, 1988). This implicates the β-adrenergic receptor in the stimulatory effect of catecholamines on CRH secretion. Norepinepherine can also increase adrenocortical secretion through an α_1-adrenergic receptor-mediated event (Al-Damluji *et al.*, 1987; Plotsky, 1987; Calogero *et al.*, 1988b; Al-Damluji *et al.*,

1990). However, this response is thought to be the result of an increased secretion of vasopressin (a potent CRF) from the hypothalamus rather than CRH (Al-Damluji *et al.*, 1990). Activation of α_2-adrenergic receptors results in an inhibition of CRH secretion (Al-Damluji, 1988). This is believed to be the result of presynaptic binding of α_2-receptor agonists causing a decrease in catecholamine release (Smythe *et al.*, 1983).

Several studies have demonstrated that activation of central $5HT_1$ and $5HT_2$ serotonin receptors enhances HPA activity (Szafarczyk *et al.*, 1979; Bagdy *et al.*, 1989; Golden *et al.*, 1989; Alper, 1990; Di Sciullo *et al.*, 1990; Fuller, 1990; Gartside and Cowen, 1990; Matsuda *et al.*, 1990; Bagdy *et al.*, 1991). *In vitro* studies report increases in bioassayable CRF (Jones and Hillhouse, 1977; Buckingham and Hodges, 1979) and in CRH (Nakagami *et al.*, 1986; Calogero *et al.*, 1989; Hillhouse and Milton, 1989; Spinedi and Gaillard, 1991) following administration of serotonin to hypothalamic explants or long term hypothalamic cultures (Nakagami *et al.*, 1986; Calogero *et al.*, 1989). In addition, serotonin has been shown to increase CRH secretion *in vivo* (Gibbs and Vale, 1983). Thus, strong evidence exists implicating a stimulatory effect of serotonin on HPA function at the level of the hypothalamus.

In vivo studies demonstrate that GABA and GABA receptor agonists inhibit and GABA receptor antagonists enhance stress-induced HPA activity (Makara and Stark, 1974). GABA receptor antagonists have also been shown to stimulate adrenocortical secretion under basal conditions (Makara and Stark, 1974). Similarly,

intracerebroventricular injection of GABA and GABA receptor agonists increased hypothalamic CRH secretion (Makara and Stark, 1974). Furthermore, stimulated release of both bioassayable corticotropin-releasing activity and CRH (as measured by radioimmunoassay) were inhibited by GABA *in vitro* (Jones and Hillhouse, 1977; Buckingham and Hodges, 1979; Calogero *et al.*, 1988c; Tsagarakis *et al.*, 1990a). There is a dense network of GABA-ergic fibers surrounding the PVN (Meister *et al.*, 1988). Also, GABA is co-stored and co-released from CRH neurons and may act on presynaptic receptors in the median eminence (Meister *et al.*, 1988). It is therefore likely that GABA is an important inhibitor of hypothalamic CRH release.

Endogenous opioids are generally believed to be inhibitory with respect to their effects on hypothalamic CRH secretion. *In vitro*, several opioids have been shown to decrease CRH secretion stimulated by neurotransmitters or depolarizing agents (Tsagarakis *et al.*, 1989a; Tsagarakis *et al.*, 1990b). Furthermore, administration of opioid receptor antagonists significantly increases basal CRH secretion. In anesthetized rats, opioid administration decreases hypophysial portal CRH and nitroprusside-induced CRH release (Plotsky, 1986). Thus, endogenous opioids may serve to maintain an inhibitory tone upon CRH release. This is believed to involve kappa- and possibly mu-, but not delta-opioid receptors (Tsagarakis and Grossman, 1990; Whitnall, 1993).

Several neuropeptides have been demonstrated to influence HPA activity and CRH secretion. *In vivo* and *in vitro* studies suggest that angiotensin II (Plotsky *et al.*, 1988), neuropeptide Y (Wahlestedt *et al.*, 1987; Haas and George, 1987; Tsagarakis

et al., 1989b), and activin (Plotsky *et al.*, 1991) are stimulatory in this respect while atrial natriuretic peptide (Takao *et al.*, 1988; Ibanez-Santos *et al.*, 1990), substance P (Chowdrey *et al.*, 1990; Faria *et al.*, 1991), and vasopressin (Plotsky *et al.*, 1984) are inhibitory. The inhibition of HPA activity by vasopressin appears paradoxical since vasopressin also has corticotropin-releasing activity. However, this may be one mechanism by which the system is kept in check.

Effects of Stress/Feedback on CRH Neurons

Stress and Glucocorticoids

Following stress, glucocorticoids act at the levels of the pituitary, hypothalamus, and extrahypothalamic loci within the brain to return HPA activity to homeostatic levels. This feedback inhibition has been a topic of intense research and has been extensively reviewed (Keller-Wood and Dallman, 1984; Jones and Gillham, 1988). This review will focus only on glucocorticoid feedback inhibition at (or above) the level of the hypothalamus. The data presented in this section are summarized in Table 2.

Corticosteroid feedback on CRH secretion has been demonstrated by the examination of the effects of adrenalectomy and/or the treatment with exogenous glucocorticoids on basal and stress-induced CRH activity. Adrenalectomy-induced increases in CRH and AVP immunoreactivity in parvocellular neurosecretory neurons are inhibited by systemic glucocorticoid replacement (Sawchenko, 1987a; Itoi *et al.*, 1987). Dexamethasone blocks increases in hypophysial portal vessel concentrations of

CRH-induced by hemorrhage stress (Plotsky and Vale, 1984), and plasma CORT levels between 80 and 120 ng/ml suppress hypotensive stress-induced increases in CRH release (Plotsky *et al.*, 1986). Conversely, adrenalectomy increases CRH and AVP concentrations in hypophysial portal plasma 3 weeks following surgery (Fink *et al.*, 1988). Pharmacological adrenalectomy using metyrapone or aminoglutathimide leads to a decrease in secretion of CRH and AVP in the initial 12-24 hours but an increase in CRH release by 72 hours (Plotsky and Sawchenko, 1987). The decrease probably reflects an initial depletion of releasable CRH pools while the subsequent increase represents the removal of inhibition by adrenal glucocorticoids. Furthermore, secretion of CRH from cultured hypothalami can be inhibited by CRH, ACTH, and dexamethasone (Calogero *et al.*, 1988d; Suda *et al.*, 1986). These studies demonstrate a hypothalamic locus for negative feedback regulation of the HPA axis but do not, however, indicate whether changes in synthesis, storage, or release of hypothalamic CRF's explain this phenomenon.

To further characterize the role of the hypothalamus in the negative feedback of the HPA axis, several investigators have studied the effects of stress, adrenalectomy, and glucocorticoids on CRH and AVP gene expression. CRH mRNA (Young *et al.*, 1986a; Kovacs and Mezey, 1987; Lightman and Young, 1989; Beyer *et al.*, 1988; Imaki *et al.*, 1991; Almeida *et al.*, 1992) and AVP mRNA (Young *et al.*, 1986b) increase in the parvocellular PVN following adrenalectomy. These increases are abolished by glucocorticoid replacement (Lightman and Young, 1989; Kovacs and Mezey 1987; Beyer *et al.*, 1988; Imaki *et al.*, 1991). This response to

Table 2. Effect of stress, adrenalectomy and glucocorticoid treatment on hypothalamic corticotropin-releasing hormone (CRH) and vasopressin (AVP).

TREATMENT	EFFECT	MEASUREMENT	REFERENCES
Adrenalectomy	increases	CRH-IR and AVP-IR in parvocellular PVN CRH mRNA and AVP mRNA parvocellular PVN	Sawchenko , 1987; Itoi et al., 1987 Young et al., 1986a; Kovacs and Mezey, 1987; Lightman and Young, 1989; Beyer et al., 1988; Imaki et al., 1991; Almeida et al., 1992; Young et al., 1986b
Glucocorticoid replacement	decreases		
Hemorrhage stress	increases	Hypophysial portal CRH	Plotsky and Vale, 1984
Dexamethasone	decreases		
Hypotensive stress	increases		Plotsky et al., 1986
Plasma corticosterone (80-120 ng/ml)	decreases		
Adrenalectomy	increases	Hypophysial portal CRH and AVP	Fink et al., 1988
Metyrapone (12-24h)	decreases		Plotsky and Sawchenko, 1987
Metyrapone (72h)	increases		
CRH	decreases	Secretion of CRH from cultured hypothalami	Calogero et al., 1988; Suda et al., 1986
ACTH	decreases		
Dexamethasone	decreases		
Hypertonic saline stress	increases	CRH mRNA levels in PVN	Lightman and Young, 1988; Lightman and Young, 1989
+Adrenalectomy	increases		
+Dexamethasone	decreases		

adrenalectomy and glucocorticoid replacement appears to be specific to the parvocellular PVN as magnocellular AVP mRNA (Young *et al.*, 1986b) and CRH mRNA in the BNST and CeA (Beyer *et al.*, 1988) are not affected. CRH mRNA and AVP mRNA also increase in the medial parvocellular region of the PVN within 4 hours of hypertonic saline stress (Lightman and Young, 1988; Lightman and Young, 1989). This may represent an increase in synthesis of CRH to replenish CRH stores released during stress. Hypertonic saline stress leads to greater increases in CRH mRNA in the PVN in the absence of negative feedback, i.e. in adrenalectomized animals as compared to intacts (Lightman and Young, 1989). Furthermore, dexamethasone-treatment attenuates basal and stress-induced increases in CRH mRNA levels (Lightman and Young, 1989). Thus, changes in CRH and AVP gene expression may be associated with the negative feedback of the HPA axis following stress.

Corticosteroid Receptors

Adrenocorticosteroids exert their effects by binding one of two intracellular receptors (for rev. see DeKloet *et al.*, 1987). These receptors have been given the designations type I and type II corticosteroid receptors. The two receptor subtypes differ from each other in their anatomical distribution, their pharmacalogical properties, and their function. The type I receptor, or mineralocorticoid receptor, is localized predominantly in the hippocampus and septum (McEwen *et al.*, 1968; Reul and DeKloet, 1985; Van Eekelen *et al.*, 1988; Arriza *et al.*, 1988; Herman *et al.*,

1989). This receptor binds with a very high affinity to CORT, aldosterone, and dexamethasone (Beaumont and Fanestil, 1983; Krosowski and Funder, 1983; Reul and DeKloet, 1985; Allen *et al.*, 1988; Brinton and McEwen, 1988). Because of this high affinity binding, the mineralocorticoid receptor is functionally important when plasma corticosteroids are low. Thus, the mineralocorticoid receptor is believed to be involved in tonic and permissive functions. These include the regulation of exploration (Veldhuis and DeKloet, 1983), food seeking (Jhanwar-Uniyal *et al.*, 1986), and basal (i.e., circadian) HPA activity (Dallman *et al.*, 1989; Ratka *et al.*, 1989).

The type II receptor, or glucocorticoid receptor, is localized in glial cells and neurons throughout the central nervous system (Fuxe *et al.*, 1985; Reul and DeKloet, 1985; Reul and DeKloet, 1986; Van Eekelen, *et al.*, 1987; Aronsson *et al.*, 1988; Sarrieau *et al.*, 1988; Van Eekelen *et al.*, 1988; Ceccatelli *et al.*, 1989c; Herman, *et al.*, 1989; Sousa *et al.*, 1989). The regions of highest concentration of glucocorticoid receptor include the hippocampus, PVN, lateral septum, central amygdala, arcuate nucleus, and supraoptic nucleus. The glucocorticoid receptor also binds CORT and dexamethasone but with lower affinity than the mineralocorticoid receptor (Krosowski and Funder, 1983; Reul and DeKloet, 1985; Brinton and McEwen, 1988). Because the glucocorticoid receptor binds CORT with a low affinity, this receptor is only occupied when plasma CORT levels are high. Thus, the glucocorticoid receptor is thought to be involved in the regulation of behavioral, neuroendocrine, and autonomic responses to stress and participates in the negative feedback inhibition of HPA activity

following stress (Reul and DeKloet, 1985).

Neuroanatomical Locus of Feedback Inhibition

There is significant evidence implicating the hippocampus, which contains both CORT receptor types, in the regulation of the HPA axis (for rev. see Jacobsen and Sapolsky, 1991). Most experiments indicate that the role of the hippocampus in this respect is inhibitory in nature. For example, electrical stimulation of the hippocampus decreases plasma CORT levels (Mandell *et al.*, 1963; Rubin *et al.*, 1966), and fornix transection or hippocampectomy results in increased plasma CORT levels (Nakadate and DeGroot, 1963; Wilson *et al.*, 1980). Furthermore, hippocampal stimulation inhibits and hippocampal lesion augments the CORT secretion induced by exposure to a stressor (Dupont *et al.*, 1972, Kawakami *et al.*, 1968). The hippocampus is also implicated in the termination of HPA responses to stress in that secretion of CORT persists much longer after exposure to restraint stress in rats with kainate lesions of the hippocampus (Sapolsky *et al.*, 1984a).

In addition to the role of the hippocampus in the negative feedback response of glucocorticoids, the PVN remains a probable site of direct glucocorticoid action in this respect. Immunocytochemical studies reveal the presence of glucocorticoid receptors in CRH-containing neurons in the PVN (Ceccatelli *et al.*, 1989c). Furthermore, unilateral implantation of dexamethasone into the PVN inhibits the adrenalectomy-induced increases in CRH and AVP immunoreactivity (Kovacs *et al.*, 1986; Sawchenko, 1987b) and in CRH mRNA (Kovacs and Mezey, 1987) on the

implanted side. Interestingly, these studies reported that implantation of dexamethasone into the hippocampus resulted in a slight decrease in the adrenalectomy-induced effects on CRH and AVP immunoreactivity (Kovacs *et al.*, 1986) while no effect was seen on CRH mRNA (Kovacs and Mezey, 1987).

The Hypothalamo-Pituitary-Gonadal Axis

The hypothalamo-pituitary-gonadal axis is an essential element of reproductive function. A subset of hypothalamic neurons synthesize gonadotropin-releasing hormone (GnRH) which is also known as leutenizing hormone releasing hormone (LHRH). These neurons project to the median eminence where they secrete GnRH episodically into the hypophysial portal vasculature (for rev. see Marshall *et al.*, 1992; Pfaff *et al.*, 1994). Gonadotropin-releasing hormone stimulates gonadotroph cells of the anterior pituitary to synthesize and secrete leutenizing hormone (LH) and follicle-stimulating hormone (FSH). In the male, leutenizing hormone stimulates Leydig cells of the testes to produce and secrete testosterone. Testosterone is necessary for spermatogenesis, reproductive behavior, and the development of male secondary sex characteristics. In addition, testosterone feeds back on the brain and pituitray to reduce HPG axis activity (Petraglia *et al.*, 1984; Bhanot and Wilkinson, 1985; Gabriel *et al.*, 1986; Wehrenberg *et al.*, 1989; Kalra *et al.*, 1990; Sahu *et al.*, 1990; Huang and Harlan, 1993).

Products of the HPA axis have been shown to inhibit reproductive function. Several studies have demonstrated that CRH attenuates hypothalamic GnRH secretion

(Rivier and Vale, 1984; Rivier and Vale 1985b; Rivier *et al.*, 1986). Furthermore, ACTH and corticosterone act on the hypothalamus, pituitary, and gonads to decrease HPG axis activity (Vreeburg *et al.*, 1984; Kamel and Kubajak, 1987; Mann *et al.*, 1987). Moreover, products of the HPA axis suppress reproductive behaviors (Plas-Rose and Aron, 1981; Sirinathsinghji *et al.*, 1983; Armstrong, 1986; Sirinathsinghji *et al.*, 1986).

Effects of Gonadal Steroids on the HPA Axis

Previous studies have demonstrated a significant sex difference in circulating CORT levels and in the CORT response to stress with females having higher levels than males (Kitay, 1961). Since then, several studies have focused on the role of estrogen in this sex difference (Kitay, 1963; Telegdy *et al.*, 1964; Raps *et al.*, 1971; Pollard *et al.*, 1975; Ramaley, 1976; Buckingham *et al.*, 1978; Phillips and Poolsanguan, 1978; Burgess and Handa, 1992). These studies demonstrate that basal CORT levels are decreased in ovariectomized rats as compared to ovariectomized rats receiving estrogen replacement (Kitay, 1963; Telegdy *et al.*, 1964; Ramaley, 1976; Lesniewska *et al.*, 1990). In addition, basal CORT as well as stress-induced CORT and ACTH levels vary with the estrous cycle (Raps *et al.*, 1971; Pollard *et al.*, 1975; Buckingham *et al.*, 1978; Phillips and Poolsanguan, 1978; Viau and Meaney, 1991a). ACTH and CORT levels are greatest on proestrous when estrogen levels are highest.

A number of studies have suggested that estrogen influences HPA activity, at least in part, at the level of the PVN. Chronic estrogen treatment of ovariectomized

female rats significantly reduced hypothalamic CRH content as determined by radioimmunoassay of hypothalamic extracts (Haas and George, 1988). A subsequent study suggested that this decrease in hypothalamic CRH content was the result of an inhibition of CRH synthesis (Haas and George, 1989). CRH mRNA in the parvocellular PVN has also been shown to vary across the estrous cycle (Bohler *et al.*, 1990). CRH mRNA is elevated on the afternoon of proestrous, at the time of the estrogen-induced midcycle surge of luteinizing hormone.

Fewer studies have focused on the effects of testosterone on the HPA axis. Gonadectomy of the male rat and hamster leads to enhanced levels of CORT correlating with increases in ACTH bioactivity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971). Following foot shock stress or exposure to a novel environment, plasma CORT and ACTH levels are higher in gonadectomized male rats as compared to intact animals (Handa *et al.*, 1994). Gonadectomized rats receiving replacement testosterone (T) or the non-aromatizable androgen, dihydrotestosterone (DHT), have post-stress plasma CORT and ACTH levels similar to that seen in intact animals (Handa *et al.*, 1994). These data suggest that, in contrast to the effects of estrogen, androgens act to inhibit the CORT and ACTH responses to stressful stimuli.

Gonadal Regulation of AVP Expression

Aside from the neurohypophysial neurons of the PVN that secrete AVP into the portal vasculature, a number of AVP-immunoreactive neuronal pathways have been demonstrated in the rat brain (Buijs, 1978). These AVP fibers arise from AVP-

IR cell bodies in the PVN (DeVries and Buijs, 1983; Sawchenko and Swanson, 1982), BNST (DeVries and Buijs, 1984), medial amygdala (MeA; Caffe *et al.*, 1987), and suprachiasmatic nucleus (Hoorneman and Buijs, 1982).

A sex difference in AVP fiber density has been demonstrated. The density of AVP fibers in the lateral septum and lateral habenular nucleus is higher in males than in females (DeVries *et al.*, 1981). Lesioning and tracing studies indicate that the origin of these fibers probably lies in the BNST (DeVries and Buijs, 1984). This sex difference is dependent on the neonatal presence of testosterone (DeVries *et al.*, 1983). In the adult animal, androgens appear to maintain this sex difference since gonadectomy of male rats has been shown to lead to a disappearance of AVP-IR cell bodies and AVP mRNA in the BNST and MeA without affecting AVP-IR cell bodies in the PVN (DeVries *et al.*, 1985; Miller *et al.*, 1992). Furthermore, androgen receptor immunoreactivity has been localized within AVP-expressing cells of the BNST and MeA (Zhou *et al.*, 1994).

Similarly, AVP fiber density in several brain regions including the MeA, lateral septum, and lateral habenular nucleus has been shown to decrease in the aged rat (Fliers *et al.*, 1985). In the rat, plasma T levels decline with age (Kaler and Neaves, 1981). When plasma T levels in aged rats were restored to that of young animals AVP fiber density was also restored (Goudsmit *et al.*, 1988). This evidence coupled with evidence that androgen and estrogen receptors are found within neurons of the BNST and MeA (Wood *et al.*, 1992; Wood and Newman, 1993; Don Carlos *et al.*, 1991; Tobet *et al.*, 1993) supports a role for gonadal hormones in the regulation

of AVP expression in some brain regions.

While these data demonstrate that androgens can regulate neuropeptides, changes in the AVP system would not explain changes in the CORT and ACTH responses to stress noted in the castrated male rat. DeVries, *et al.* (1985) report that castration did not affect the number of AVP-IR cell bodies in the PVN, and changes in AVP-IR cell bodies in other brain regions were in the wrong direction to explain androgen influences on the HPA response to stress.

Mechanism of Steroid Action

Signal Transduction

Gonadal steroids initiate changes in cellular function via specific intracellular receptors which act as ligand-responsive transcription factors (for rev. see Blaustein, 1985; Evans, 1988). The gonadal and adrenal steroid receptors have been grouped in a "superfamily" of intracellular receptors due to the similar function and high degree of sequence homology among these receptors (for rev. see Evans, 1988). This receptor superfamily includes receptors for vitamin D, thyroid hormone, retinoic acid, and the retroviral oncogene product, v-erb A. (The mechanism of steroid receptor action is depicted in Figure 6).

Previously, it was believed that unoccupied steroid receptors may be localized in the cytoplasm and may need to be translocated into the cell's nucleus following ligand binding. Recent data, however, suggest that unoccupied receptors may be localized within the cell nuclei (Sheridan *et al.*, 1979; King and Greene, 1984;

Welshons *et al.*, 1984; Gasc *et al.*, 1989; Zhuang *et al.*, 1992; Pekki *et al.*, 1992). These unbound receptors are found complexed with two 90 kiloDalton heat shock protein (hsp90) molecules (Catelli *et al.*, 1985; Sanchez *et al.*, 1985; Mendel and Orti, 1988). The function of hsp90 may be related to protein folding, transport, and/or stabilization (Pratt, 1990). Other heat shock proteins, hsp59 and hsp70, also participate in this multiprotein complex (Sanchez, 1990; Muller and Renkawitz, 1991) although their functions are currently unknown. The inactive complex is loosely bound to nuclear chromatin.

When the hormone binds to its high affinity binding site on the receptor molecule, hsp90 dissociates. The receptor-ligand complex can then become tightly bound to DNA. When bound to its ligand, an intracellular receptor alters gene expression of specific steroid-responsive genes. Steroid-responsive genes contain short (about 20 base pairs) cis-acting sequences or hormone response elements (HREs) that are required for hormonal activation of the gene (Govindan *et al.*, 1982; Scheidereit *et al.*, 1983; Karin *et al.*, 1984). These HREs function in a position- and orientation-independent fashion as transcriptional enhancers (Chandler *et al.*, 1983). Thus, transcriptional regulation depends on the binding of hormone-receptor complexes to HRE sites on DNA. Identification of HRE sequences reveals an apparent dyad symmetry implying that HREs interact with receptor dimers (Waterman *et al.*, 1988; Chalepakis *et al.*, 1990).

Hormone-receptor coupling is also necessary for induction of the receptors trans-activation function (Webster *et al.*, 1988). The ligand-receptor complex alters

gene transcription by interacting with other components regulating transcription. For example, ligand-activated steroid receptors have been shown to interact with the factor which binds to a consensus sequence, the TATA box, found within many promoter regions (Ptashne, 1988; Ham *et al.*, 1988).

Steroid receptors have been shown to interact with other transcription factors as well (Schule *et al.*, 1988; Strahle *et al.*, 1988). For example, binding of the glucocorticoid receptor to its HRE enhances the transcription induced by the binding of another factor to its binding site, a CACCC-box (Schule *et al.*, 1988). This synergism is dependent upon a distance of 10 base pairs between the binding sites. Ten base pairs corresponds to one turn of the double helix. Thus, a stereo-specific alignment and a protein-protein interaction between the receptor-ligand complex and the other transcription factor is likely.

In addition to the positive regulation of gene expression outlined above, steroid hormone receptors can negatively regulate gene expression. Various mechanisms by which intracellular receptors repress gene expression have been described. First, binding of a receptor-ligand complex to its HRE can displace a positive regulatory factor (Akerbloom et al., 1988; Guertin et al., 1988; Sakai et al., 1988; Beato, 1989; Drouin et al., 1989). For example, the occupied glucocorticoid receptor represses expression of the human glycoprotein α-subunit gene because the glucocorticoid response element overlaps a functional cAMP response element (Akerbloom et al., 1988). Thus, binding of the glucocorticoid receptor to its HRE sterically hinders the binding of the positive transcription factor to the cAMP response element.

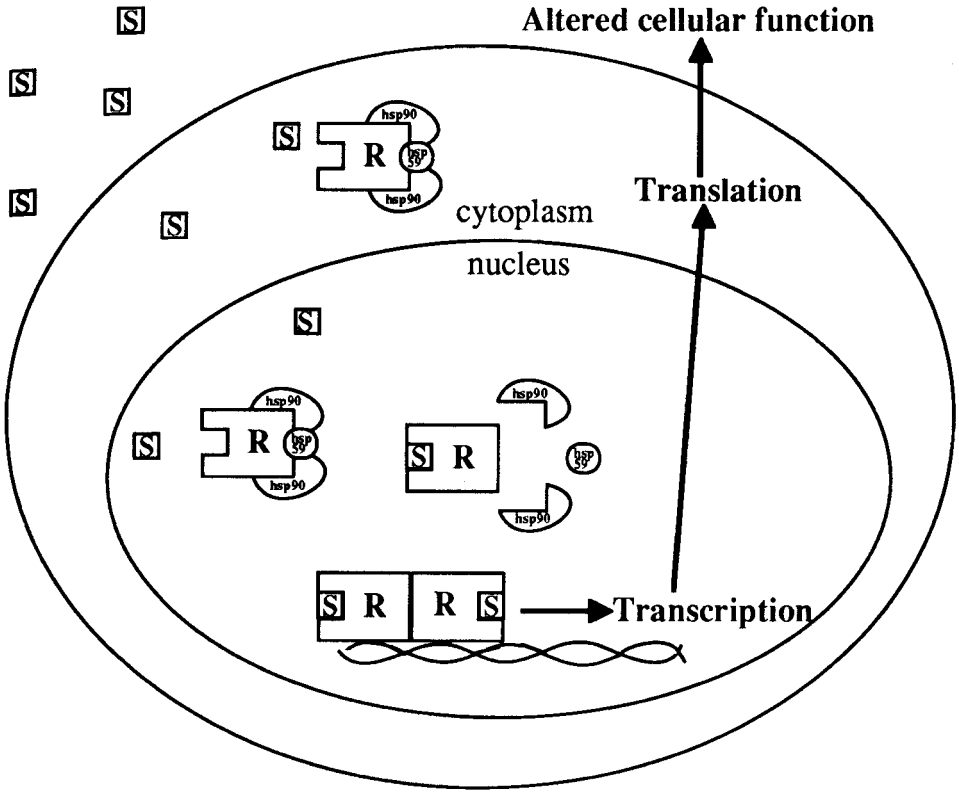


Figure 6. Pathway of signal transduction of intracellular steroid receptors. The steroid (S) diffuses through the cell membrane where it binds to the receptor (R) which is complexed to heat shock proteins (hsp). The hsp molecules dissociate, and the steroid-receptor complex binds DNA as a homodimer. Binding of the receptor-ligand complex can lead to transcription of mRNA's, translation of proteins, and ultimately an alteration of cellular function.

A second mechanism by which steroid receptors negatively regulate gene expression involves the regulation of AP-1 mediated induction of gene expression (see Figure 7). AP-1 is a transcriptional activator composed of protein dimers of jun and fos gene family products. Both Jun-Fos heterodimers and Jun-Jun and Fos-Fos homodimers are capable of binding the AP-1 site and inducing gene expression (Diamond *et al.*, 1990). However, the Jun-Fos heterodimer binds with higher

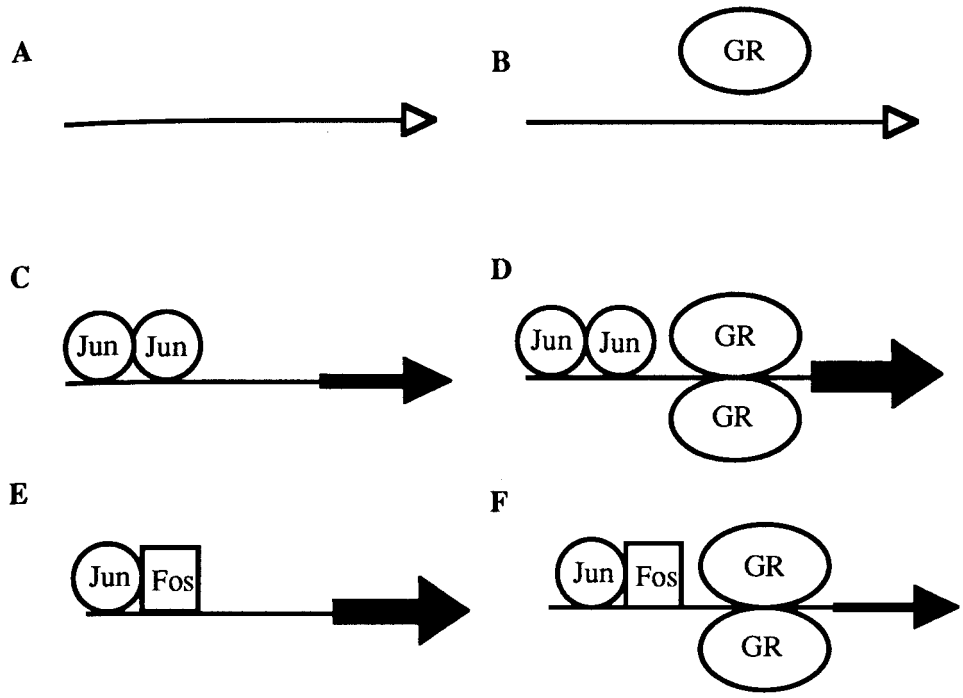


Figure 7. A model of glucocorticoid regulation of AP-1-mediated transcription of the proliferin gene. In each panel the solid line represents the DNA surrounding the promoter. The different sized arrows depict relative levels of transcriptional activity. In the absence of AP-1 activity the glucocorticoid-receptor complex (GR) does not influence transcription (A,B). The Jun homodimer activates transcription (C). The GR enhances Jun homodimer-induced transcription (D). The Jun-Fos heterodimer strongly activates transcription (E). However, the GR reduces heterodimer-induced transcription (F); (adapted from Diamond *et al.*, 1990).

affinity and more strongly activates gene expression compared to the homodimers. Glucocorticoids can both positively and negatively regulate the AP-1 mediated induction of the proliferin gene (Diamond et al., 1990). The positive and negative regulation of gene expression by glucocorticoids depends upon the intracellular ratio of Jun and Fos. The synthetic glucocorticoid, dexamethasone, enhances the expression of proliferin activated by Jun homodimers. However, dexamethasone reduces Fos homodimer- and Fos/Jun heterodimer-induced gene expression. Thus,

interaction of the glucocorticoid receptor with Jun homodimers results in positive gene regulation while interaction of the glucocorticoid receptor with Fos/Jun heterodimers results in negative regulation of the gene (Diamond et al., 1990). Recently, it was demonstrated that binding of the glucocorticoid receptor to the transcription factor, AP-1, results in a repression of AP-1 stimulated expression of the collagenase gene (Jonat et al., 1990). This direct interaction of a receptor-ligand complex with a transcription factor occurs in the absence of DNA binding. Thus, it appears that an AP-1/glucocorticoid receptor complex is formed which is unable to trans-activate.

The mechanism by which specific hormonal regulation of gene transcription is attained *in vivo* has been a fundamental question in the field of steroid receptor biology. This is because *in vitro* several receptors can bind the same DNA binding sites (Cato *et al.*, 1988; Ham *et al.*, 1988). For example, glucocorticoids and androgens recognize a common HRE. Recent studies have indicated the presence of a complex, 120 base pair enhancer of the mouse sex-limited protein (Slp) gene that is activated exclusively by androgens and not by glucocorticoids in transfection (Adler *et al.*, 1991; Adler *et al.*, 1992; Adler *et al.*, 1993). Hormonal specificity is enforced by non-receptor elements within the enhancer (Adler *et al.*, 1992). Androgen-specific activation occurs when ligand-activated androgen receptor binds the consensus HRE site and interacts with a specific factor on the enhancer to activate transcription (Adler *et al.*, 1992). Glucocorticoid receptor may bind the HRE but is unable to transactivate (Adler *et al.*, 1992). Selectivity appears to be dependent upon the amino terminus of the receptor (Adler *et al.*, 1992).

Receptor Structure

Comparisons of sequences and functions of the various members of the steroid receptor superfamily demonstrate a common basic structure for steroid receptors (for rev. see Evans, 1988; see Figure 8). The trans-activation domain (region A/B) lies closest to the amino-terminus of the receptor protein. This domain is highly variable among the receptors and is responsible for the trans-activation function outlined above. This region is also thought to play a role in gene recognition specificity (Evans, 1988; Adler *et al.*, 1992). The DNA-binding domain (region C) is a 66-68 amino acid region rich in cysteine residues which arrange themselves around zinc ions forming the "zinc fingers" involved in binding to DNA (for rev. see Evans and Hollenberg, 1988). The hinge region (region D) is involved in protein folding following hormone-receptor coupling. The ligand-binding domain (region E) is a 220-250 amino acid region which forms a hydrophobic pocket for steroid binding. This region is also the site of hsp90 binding. Surprisingly, neither the ligand-binding domain nor the hormone is necessary for DNA binding. Rather, the ligand-binding domain prevents the otherwise constitutive binding of the DNA binding domain to the HRE, and ligand-receptor coupling relieves this inhibition (Godowski *et al.*, 1987; Hollenberg *et al.*, 1987; Waterman *et al.*, 1988). The region closest to the carboxy-terminus of the receptor (F) is a highly variable region of unknown function.

The Androgen Receptor

The recent cloning and sequence analysis of androgen receptor (AR) cDNA reveals a structure similar to other steroid receptors (Lubahn *et al.*, 1988a; Chang *et*

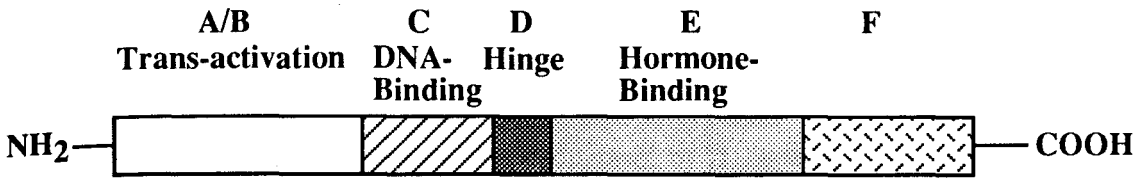


Figure 8. Structural and functional representation of steroid receptors. Receptors are divided into six regions (A-F). The A/B region is located at the amino terminus and is responsible for the trans-activation function of the receptor. The C region is a highly conserved region responsible for DNA binding. The hinge region (D) is involved in protein conformational changes. The E region forms a hydrophobic pocket for ligand binding. The carboxy terminus of the protein (region F) is highly variable and of unknown function (adapted from Evans, 1988).

al., 1988; for rev. see Janne et al., 1993). The deduced amino acid sequences of rat and human AR reveal 902 and 919 amino acid proteins, respectively (Tan *et al.*, 1988; Lubahn *et al.*, 1988b). The DNA-binding domain of the human AR shares greatest homology with progesterone, mineralocorticoid, and glucocorticoid receptors (Lubahn *et al.*, 1988a) The amino terminal end (which is characteristically variable among steroid receptors) shows little similarity to other receptor sequences (Lubahn *et al.*,1988b). Furthermore, rat and human AR share complete amino acid sequence identity in their DNA- and ligand-binding domains (Lubahn *et al.*, 1988b; Tan *et al.*, 1988).

Aromatization of Testosterone

In the brain, testosterone can influence cellular function differentially

depending on its intracellular fate (for rev. see McEwen *et al.*, 1982). There are three alternative intracellular fates for testosterone (see Figure 9). Once testosterone has passed through the neuronal membrane, it can bind directly to AR or it can be reduced by the intracellular enzyme, 5α-reductase, to DHT which also binds with high affinity to AR. Alternatively, testosterone can be converted to 17β-estradiol by the intracellular aromatase enzyme (Weisz and Gibbs, 1974; Naftolin *et al.*, 1975). This 17β-estradiol can then bind to estrogen receptors (ER) and will thus influence cellular function in an alternate manner. Thus, the way in which testosterone will affect neuronal function in specific brain regions depends on the presence or absence of AR, ER, and the aromatase enzyme.

A recent study suggests that a large proportion of the aromatase-IR cells in the quail brain do not contain immunoreactive ER (Balthazart *et al.*, 1991). In fact, significant amounts of aromatase activity are found in synaptosomes purified by differential centrifugation (Steimer, 1988; Schlinger and Callard, 1989). Also, aromatase immunoreactivity is observed at the light microscopic level in fibers and punctate structures (Balthazart et al., 1990) and at the electron microscopic level in synaptic boutons (Naftolin et al., 1990). It has been hypothesized that aromatase might produce estrogens which act at the synaptic level as neurohormones or neuromodulators (Balthazart and Foidart, 1993). Although this is still quite speculative, it is possible that aromatization of testosterone may occur in glia and in neurons that do not contain ER. The generated 17β-estradiol may then transfuse to ER containing cells.

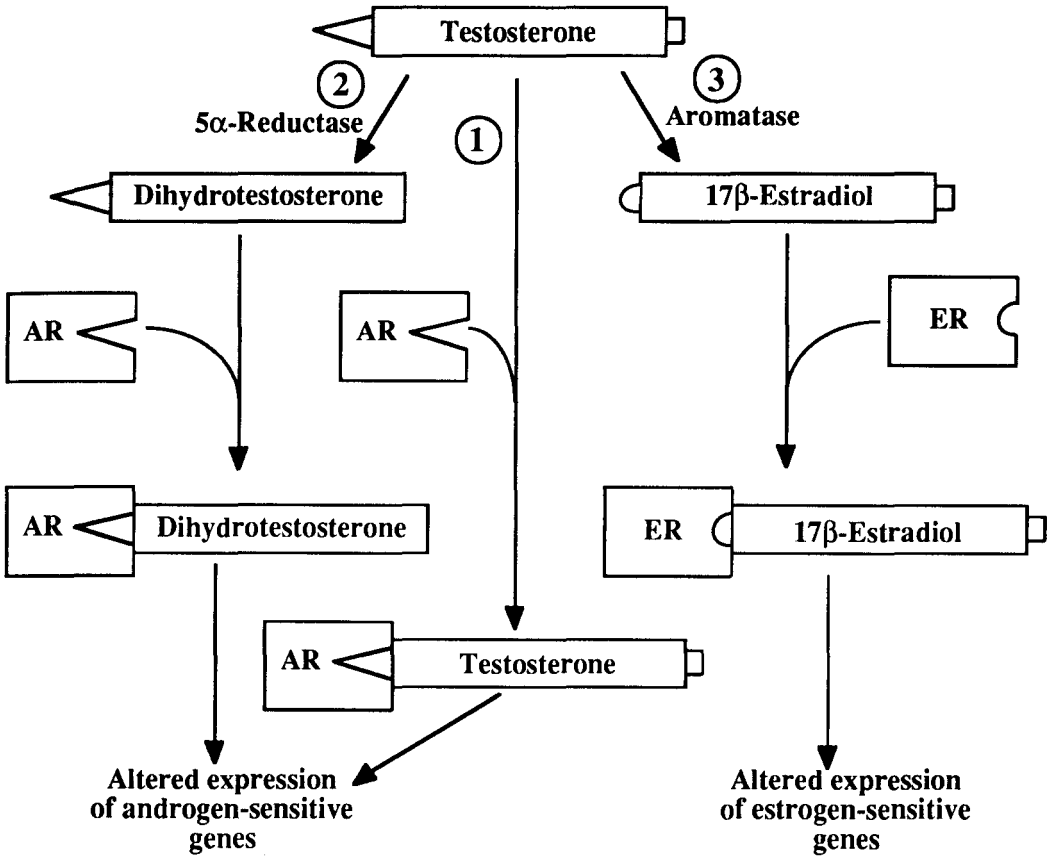


Figure 9. The intracellular fates of testosterone. After diffusing through the cell membrane, testosterone can either (1) bind the androgen receptor or (2) be reduced to dihydrotestosterone and bind the androgen receptor. Alternatively, testosterone can (3) be aromatized to 17β-estradiol and bind the estrogen receptor.

Distribution of Androgen and Estrogen Receptors

Steroid autoradiographic studies using ^{3}H-DHT (Sar and Stumpf, 1977; Sheridan 1978; Doherty and Sheridan, 1981; Wood et al., 1992) revealed androgen target cells in the preoptic area, PVN, periventricular, ventromedial, and arcuate nuclei of the hypothalamus in rat, mouse, and hamster. High DHT uptake was also noted in the amygdala, lateral septum, BNST, and hippocampus. More recently, immunohistochemistry and ISHH have revealed similar distributions of AR immunoreactivity (Wood and Newman, 1993) and AR mRNA (Simerly *et al.*, 1990). It has been reported that the distribution of AR mRNA and CRH in the PVN do not overlap (Simerly *et al.*, 1990). However, this is contradicted by observations of AR mRNA within this region (Handa, unpublished data).

The distributions of estrogen-concentrating cells, ER-IR cells, and ER mRNA-expressing cells have also been described previously (Pfaff and Keiner 1973; Loy *et al.*, 1988; Rhodes *et al.*, 1982; Rhodes *et al.*, 1981; Cintra *et al.*, 1986; Don Carlos *et al.*, 1991; Pelletier *et al.*, 1988; Simerley *et al.*, 1990). The distribution of these estrogen sensitive neurons significantly overlaps the distribution of AR containing cells (Sar and Stumpf 1977; Sheridan, 1978; Doherty and Sheridan, 1981; Wood *et al.*, 1992; Wood and Newman 1993; Simerly *et al.*, 1990). However, important differences exist between the distribution of AR and ER. For example, ER immunoreactivity and ER mRNA are abundant within the dorsal zone of the medial parvocellular PVN where CRH neurons are localized (Don Carlos *et al.*, 1991; Simerly *et al.*, 1990). Also, in the ventral zone of the medial parvocellular PVN

which contains many cells that project to autonomic regions of the brain stem and spinal cord, AR mRNA- but not ER mRNA-containing cells have been observed (Simerly *et al.*, 1990). However, this contradicts earlier reports of estrogen concentrating cells in this region (Rhodes *et al.*, 1981).

In the rat the aromatase enzyme is concentrated in several brain regions that contain ER such as the preoptic area, medial basal hypothalamus, lateral septum, BNST, and amygdala (Naftolin *et al.*, 1975; Selmanoff *et al.*, 1977; Lauber *et al.*, 1993; Jakab *et al.*, 1993). Thus, testosterone could influence cells in these areas through it's aromatization to 17β-estradiol and subsequent binding to ER.

Steroid hormones can influence the sensitivity of target cells to further hormonal stimulation. A steroid hormone accomplishes this by regulating the expression of genes encoding its own receptor, other steroid receptors, or the aromatase enzyme. Androgen receptor and estrogen receptor provide examples of homologous regulation of receptor expression (Saceda *et al.*, 1988; Tan *et al.*, 1988; Ree *et al.*, 1989; Shupnick *et al.*, 1989; Berthois *et al.*, 1990; Don Carlos *et al.*, 1990; Quarmby *et al.*, 1990; Shan *et al.*, 1990; Sanborn *et al.*, 1991; Takeda *et al.*, 1991; Lauber *et al.*, 1992; Lisciotto and Morrell 1993). Examples of heterologous regulation of receptor expression include the regulation of androgen receptor and progestin receptor by estrogen (Handa *et al.*, 1987; Handa and Rodriguez, 1991; Blaustein *et al.*, 1988; Don Carlos and Morrell, 1989). Furthermore, the induction of aromatase activity by androgens represents a limiting factor in the activation of male copulatory behavior and has been demonstrated in several species (Connolly *et al.*,

1990; Hutchinson *et al.*, 1990: Weaver and Baum, 1991; Harada *et al.*, 1992; Fadem *et al.*, 1993; Harada *et al.*, 1993; Resko *et al.*, 1993; Connolly *et al.*, 1994). Thus, steroid hormones can influence the sensitivity of target cells by altering the concentration of steroid receptors and the aromatase enzyme.

Implications and Hypothesis

In mammals, the biological reaction to anxiogenic stimuli involves well-characterized neuroendocrine, autonomic, and behavioral changes that serve to protect the integrity of the organism. The neuroendocrine system responds with increases in plasma ACTH, corticosterone, and prolactin. Autonomic outputs elicit very rapid and specific changes including increases in blood pressure and heart rate. In the rat, changes in behavior include an increase in freezing and a decrease in rearing.

Previous studies indicate that androgens affect the HPA axis. Gonadectomized male rats have increased CORT and ACTH responses to physical or psychological stressors as compared to intact animals (Handa *et al.*, 1994). Furthermore, treatment of castrates with testosterone or DHT restores the CORT and ACTH responses to these stressful stimuli to that of intact rats. It is important to determine whether circulating androgens influence other aspects of the stress response. **Thus, our hypothesis is that in the male rat, circulating androgens modulate the neuronal circuitry responsible for mediating neuroendocrine and behavioral responses to stressful stimuli.** Dysfunction of the stress response is believed to lead to adjustment disorders such as depression and panic disorder. These disorders occur much more

often in females than in males (Weissman and Klerman, 1977; Katschnig and Amering, 1990). Androgens may play a role in protecting a male from disfunction of the stress response. Consequently, the following experiments were designed to determine whether androgens influence CRH content, CRH-immunoreactivity, and CRH mRNA levels in the PVN. It will be determined whether CRH-containing neurons of the PVN contain androgen receptor, thus indicating whether androgens can act within these cells to alter hypothalamic CRH levels. Finally, the effects of androgens on neuroendocrine, autonomic, and behavioral responses to conditioned stress will be examined.

CHAPTER III

ANDROGENS INFLUENCE HYPOTHALAMIC CORTICOTROPIN-RELEASING HORMONE (CRH) AND CRH-IMMUNOREACTIVITY FOLLOWING GONADECTOMY

Summary

To characterize the effect of androgens on the hypothalamo-pituitary-adrenal (HPA) axis we examined the regulation of corticotropin-releasing hormone (CRH) following gonadectomy and hormone replacement. Three month-old, male Fischer 344 (F344) rats were gonadectomized (GDX) or sham gonadectomized (SHAM). Control animals remained intact. Animals were sacrificed 1, 4, 7, 10, or 21 days following surgery. GDX rats had significantly elevated ($p < 0.05$) levels of hypothalamic CRH 21 days after surgery compared to intact and sham-operated rats. In a second study, three month-old male F344 rats were gonadectomized and treated with the non-aromatizable androgen, dihydrotestosterone (DHT) using a Silastic capsule containing crystalline DHT propionate subcutaneously implanted in each animal's back. Control animals were gonadectomized and sham-treated (GDX) or left intact (INT). Three weeks following GDX, CRH levels in the hypothalamus of GDX

rats showed a significant increase ($p < 0.05$) compared to intact animals. DHT-treatment at the time of gonadectomy prevented this increase. CRH or arginine vasopressin (AVP) immunoreactivity was examined using immunocytochemistry. The number of CRH-immunoreactive (IR) cells in the paraventricular nucleus (PVN) of gonadectomized, DHT-treated animals was significantly decreased ($p < 0.05$) compared to GDX rats. No differences were seen between treatment groups in CRH-IR cell numbers in the bed nucleus of the stria terminalis or the central amygdaloid nucleus or in AVP-IR cell numbers in the PVN. These data demonstrate that long term castration increases hypothalamic CRH content and CRH-IR cell numbers in the PVN by removal of an androgen-dependent repression.

Introduction

The hypothalamo-pituitary-adrenal (HPA) axis is a principle component of an animal's adaptive response to environmental perturbations. Neurons located in the parvocellular region of the paraventricular nucleus (PVN) of the hypothalamus project to the median eminence where they secrete a 41-amino acid peptide, corticotropin-releasing hormone (CRH), into the portal vasculature. Corticotropin-releasing hormone then stimulates corticotrophs of the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) which in turn stimulates the secretion of corticosterone (CORT) from the adrenal cortex.

Products of the HPA axis have been shown to inhibit reproductive function by suppressing both gonadotropin secretion (Rivier and Vale, 1984; Rivier and Vale,

1985b; Rivier *et al.*, 1986; Kamel and Kubajak, 1987) and reproductive behaviors (Plas-Rose and Aron, 1981; Sirinathsinghji *et al.*, 1983; Armstrong, 1986; Sirinathsinghji *et al.*, 1986). Conversely, gonadal hormones are known to affect HPA activity. Previous studies have demonstrated a significant sex difference in circulating CORT levels and in the CORT response to stress with females having higher levels than males (Kitay, 1961). The greater secretion in females is believed to be the result of estrogen acting to enhance HPA activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992).

In contrast, in males androgens may inhibit HPA activity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994; Viau and Meaney, 1991b). Previous studies have shown that prepuberal gonadectomy of the male rat and hamster lead to enhanced levels of CORT which correlate with increases in ACTH bioactivity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971). Consistent with these findings, we have shown that, following foot shock stress or exposure to a novel environment, plasma CORT and ACTH levels are higher in gonadectomized male rats as compared to intact animals. Furthermore, gonadectomized rats receiving replacement testosterone or the non-aromatizable androgen, dihydrotestosterone (DHT), have post-stress plasma CORT and ACTH levels similar to that seen in intact animals (Handa *et al.*, 1994). These data support the hypothesis that the sex difference in HPA activity arises as a result of the circulating gonadal steroid environment.

Several possible mechanisms could explain androgen's inhibition of HPA activity. Androgens could be inhibiting synthesis and secretion of hypothalamic CRH

(or other corticotropin-releasing factors such as arginine vasopressin, AVP), enhancing the negative feedback of the HPA axis, or decreasing anterior pituitary sensitivity to CRH. The hypothesis that androgens influence the negative feedback response is not supported because there are no differences in either concentration or affinity of type I or type II CORT receptors in the hippocampus or hypothalamus in intact versus castrated rats (Handa *et al.*, 1994). Furthermore, anterior pituitary responsiveness to CRH does not appear to be altered following gonadectomy of male rats (Handa *et al.*, 1994). The possibility remains that androgens could be influencing hypothalamic CRH synthesis and secretion.

In order to determine whether androgens affect the HPA axis by inhibiting hypothalamic CRH levels, we examined the effect of the non-aromatizable androgen, DHT, on hypothalamic CRH content and CRH-immunoreactive (IR) cell numbers within the PVN.

Materials and Methods

Animals

Three month old, male Fischer 344 rats (F344, Harlan/Sprague Dawley, Indianapolis, IN) were maintained on a 12:12 light:dark schedule with lights on at 0700h. Animals were housed in temperature controlled rooms. Food and water were available ad libitum. For timecourse studies, animals were either bilaterally gonadectomized (GDX) or received a sham operation (SHAM) under ether anesthesia. Control animals remained intact (INT). Animals were sacrificed 1, 4, 7, 10, or 21

days after surgery between 0900 and 1100h. Animals were sacrificed by decapitation immediately following removal from their home cage. Hypothalami were dissected for evaluation of CRH content. To study the effects of androgen-replacement in castrates, 3 month-old male F344 rats were bilaterally gonadectomized under ether anesthesia. Gonadectomized animals received either hormone replacement using a 2.5 cm Silastic capsule (0.07" I.D., 0.125" O.D.; Dow Corning, Midland, MI) containing crystalline dihydrotestosterone propionate (DHT) subcutaneously implanted in each animal's back (GDX+DHT) or a sham incision (GDX). Control animals were left intact (INT). Animals were sacrificed 21 days after surgery between 0900 and 1100 h. For measurement of hypothalamic CRH content, animals were sacrificed by decapitation immediately following removal from their home cage. Trunk blood was collected into tubes containing 300 μl 0.3 M ethylenediaminetetraacetic acid (EDTA). Plasma was frozen (-80°C) until assayed for testosterone and DHT. For immunocytochemical studies, animals were anesthetized with a lethal dose (0.13 mg/kg) of sodium pentabarbital and perfused through the ascending aorta with 4% buffered paraformaldehyde. Blood was taken by cardiac puncture just prior to perfusion and added to tubes containing 300 μl 0.3 M EDTA. Animals were NOT treated with colchicine prior to sacrifice because the resulting accumulation of CRH could mask treatment effects.

CRH Radioimmunoassay (RIA)

Hypothalami were dissected according to the following landmarks: the optic

chiasm rostrally, the mammillary bodies caudally, the roof of the third ventricle dorsally, the infundibulum ventrally, and the hypothalamic sulci laterally. Tissue was homogenized in 1 ml 0.1 N HCl:2 N acetic acid (1:1), boiled for 3 minutes, and centrifuged at 2000 x g for 20 minutes. The supernatant was dessicated in a Centrivap concentrator (Labconco, Kansas City, MO) and stored at -80° C until assayed. CRH content was measured by RIA using anti-CRH serum (Peninsula Laboratories, Belmont, CA) raised against synthetic CRH (human, rat). ^{125}I-CRH (NEN, Boston, MA) was used as the tracer. Protein concentration in the pellets recovered following the 2000 x g centrifugation was determined by the method of Lowry, *et al.* (1951). The ED_{50} was 30.65 pg/tube. The assay limit of detectability was 11.8 pg/tube. The intra- and inter-assay coefficients of variance were 3.6% and 10.2%, respectively.

Immunocytochemistry

Brains were post-fixed overnight in 4% paraformaldehyde in borate buffer, and 20 μm-thick coronal sections were cut through the forebrain with a vibratome (Technical Products Intl., St. Louis, MO). Every fourth section was used for evaluation of CRH immunoreactivity. Adjacent sections were used for evaluation of arginine vasopressin (AVP) immunoreactivity. The following primary antisera were used in this study: anti-corticotropin-releasing hormone (lot #465) provided by the late Dr. T.L. O'Donohue, and anti-arginine vasopressin (48 bleed 10) provided by Dr. S.J. Watson (Univ. of Michigan, Ann Arbor). Free floating sections were

incubated with antiserum diluted at either 1:2000 (CRH) or 1:10,000 (AVP) in 0.1 M phosphate buffered saline with 0.25% Triton X-100 (Malinkrodt, Inc., Paris, KY) and 2% normal donkey serum for 18-24 hours at 4°C. Immunoreactivity was visualized using a biotinylated secondary antibody, streptavidin-horseradish peroxidase, and diaminobenzidine. Sections were mounted on gel-coated slides, and CRH-IR cells in the PVN, bed nucleus of the stria terminalis (BNST), and central amygdaloid nucleus (CeA), and AVP-IR cells in the PVN were counted without knowledge of treatment group. Labelled cells were distinguished based on the discretion of the examiner. The number of counted cells were multiplied by 4 (every fourth section was used) and corrected for double-counting errors according to Abercrombie (1946).

As a control for antisera specificity, 1 ml of each diluted antiserum was preincubated with 50 μg of its respective synthetic neuropeptide, and the sections were processed as usual to determine whether any residual staining remained. Although immunohistochemical staining was eliminated in these controls, it is possible that these antisera may recognize unidentified neuropeptides containing similar amino acid sequences. Thus, the neuropeptide immunoreactivity detected in this study is more accurately described as neuropeptide-like immunoreactivity.

Steroid Radioimmunoassays

Plasma testosterone and DHT levels were evaluated by RIA using a ^{125}I-testosterone kit (ICN Biomedicals, Inc., Casa Mesa, CA). The standard curve ranged from 5 to 500 pg/tube. Bound and free steroid were separated using a goat anti-rabbit

gamma-globulin. The RIA sensitivity limit was 1.98 pg/tube. The intra-assay coefficient of variance was 9.5%. All values were analyzed in one assay to avoid inter-assay variation.

For plasma DHT determination, steroids were extracted from samples using hexane:ethyl acetate (3:2) and separated on microcelite columns (ICN Biomedicals, Inc.) using an isooctane-ethyl acetate system. DHT was eluted from the column with 5% ethyl acetate in isooctane. Recovery of DHT was 80%. Plasma DHT was determined by RIA using a ^{3}H-dihydrotestosterone RIA kit (ICN Biomedicals, Inc.). The standard curve ranged from 10 to 500 pg/tube. Bound and free steroid were separated using dextran-coated charcoal. The RIA sensitivity limit was 30.3 pg/tube. The intra-assay coefficient of variance was 10.9%. All values were analyzed in one assay to avoid inter-assay variation.

Statistics

Time course data were analyzed by two-way analysis of variance across treatment and time (Winer, 1977). Data from DHT-replacement studies were analyzed by one-way analysis of variance (Winer, 1977). Plasma DHT levels were analyzed by a two-tailed t-test (Winer, 1977). A p value of <0.05 was considered significant. Newman-Keuls' test was used for post hoc analysis.

Results

Hypothalamic CRH Levels

Radioimmunoassayable CRH content in the hypothalamus was significantly increased ($p < 0.05$) in GDX rats as compared to intact animals 21 days after surgery (Figure 10). Sham gonadectomy had no effect on hypothalamic CRH content across any time point examined. Hypothalmic CRH content in intact male F344 rats averaged 1.6 ± 0.1 ng/hypothalamus. DHT administered continuously from the time of gonadectomy prevented the effect of GDX 21 days after surgery (Figure 11). Plasma testosterone and DHT levels are shown in Table 3. Gonadectomy reduced circulating androgens, and hormone treatment increased DHT titers.

Immunocytochemistry

Corticotropin-releasing hormone-IR cell numbers were significantly greater ($p < 0.05$) in the PVN of GDX male rats as compared to GDX animals receiving replacement DHT titers (Figure 12). When CRH-IR cell numbers in the PVN of GDX and GDX + DHT rats were expressed as a percent of the intact cell count for each immunocytochemical reaction, GDX rats had $198.1 \pm 47.3\%$ and rats in the GDX + DHT group had $63.2 \pm 12.0\%$ of the CRH-IR cell numbers seen in intact animals. No significant differences were seen in CRH-IR cell numbers in the BNST or CeA between the treatment groups. Furthermore, no significant differences were seen between treatment groups in AVP-IR cell numbers in the PVN. Representative coronal sections demonstrating CRH-IR cells and AVP-IR cells in the PVN of intact,

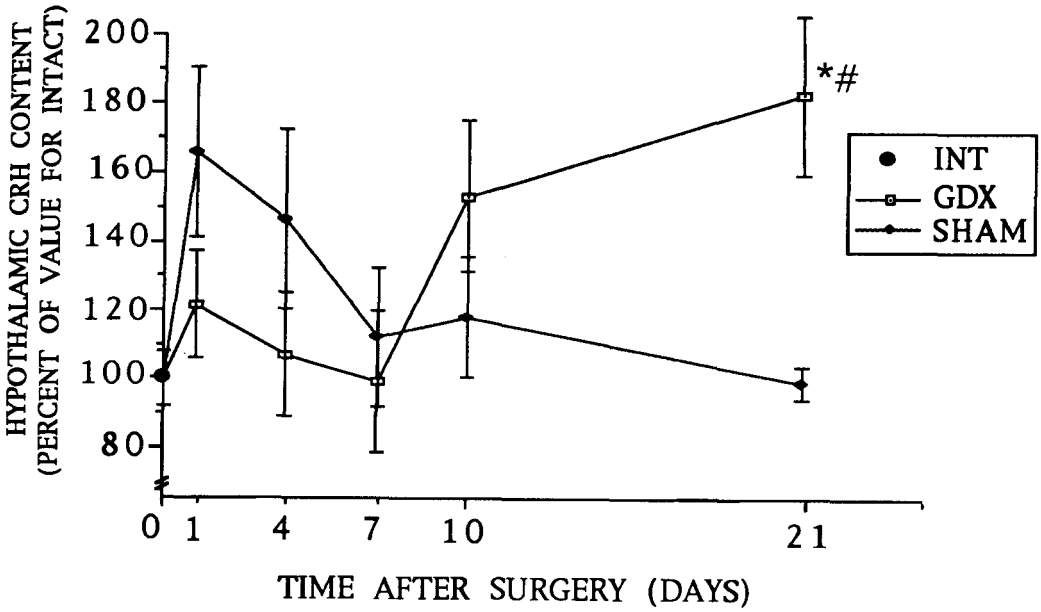


Figure 10. Hypothalamic CRH content in gonadectomized (GDX) and sham gonadectomized (SHAM) male F344 rats 1, 4, 7, 10, or 21 days following surgery. Values were determined by radioimmunoassay and expressed as a percent of the mean hypothalamic CRH content of intact male rats (INT) run with each assay. Hypothalamic CRH content of intact rats averaged 1.6 $\pm$ 0.1 ng/hypothalamus. * significant difference ($p < .05$) from intacts. # significant difference ($p < .01$) from 21-day shams. n=5-12/group. Each point represents the mean $\pm$ the standard error of the mean of values obtained from individual rats within each treatment group.

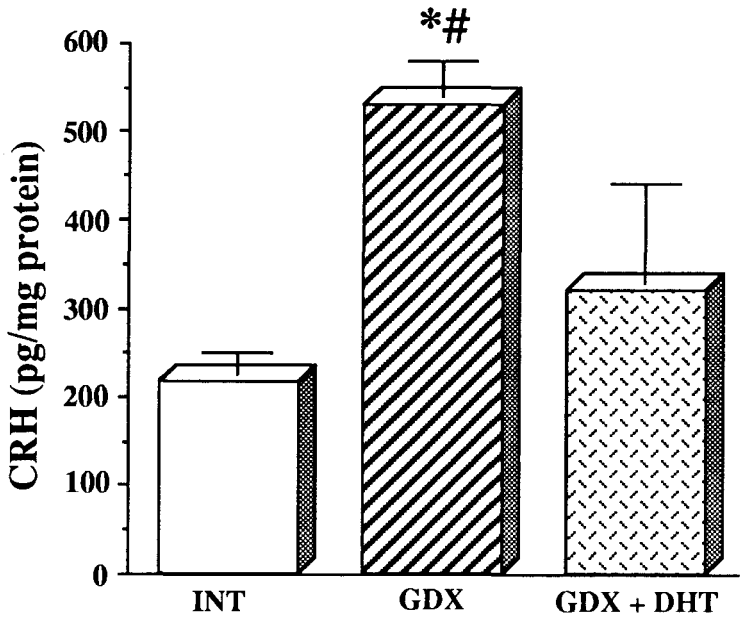


Figure 11. Hypothalamic CRH content in intact (INT), gonadectomized (GDX), and gonadectomized, dihydrotestosterone-treated (GDX + DHT) male F344 rats determined by radioimmunoassay and expressed as pg CRH/mg protein. Animals were gonadectomized 3 weeks prior to sacrifice. DHT was administered at the time of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate implanted under the skin of each animal's back. * designates groups which are significantly different ($p < 0.05$) from intact animals. # designates groups which are significantly different ($p < 0.05$) from DHT-treated animals. n=5-6/group. Each bar represents the mean $\pm$ standard error of the mean of values obtained from individual rats within each treatment group.

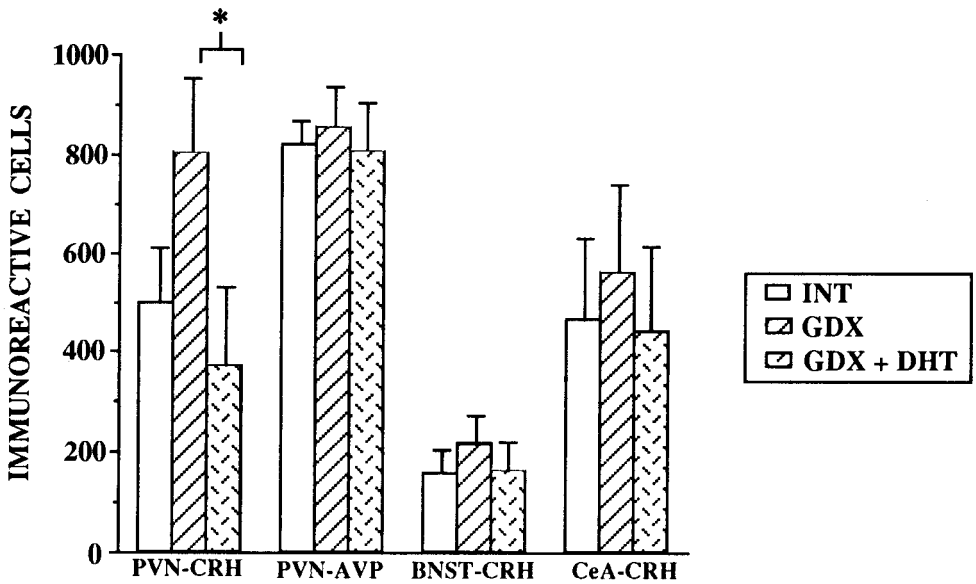


Figure 12. CRH-IR and AVP-IR cell numbers in the paraventricular nucleus (PVN), bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA) of male F344 rats. Animals were either intact (INT) or bilaterally gonadectomized under ether anesthesia. Half of the gonadectomized animals received either dihydrotestosterone (GDX + DHT) or a sham operation (GDX). DHT was administered continuously for the duration of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate subcutaneously implanted in each animal's back. * GDX and GDX + DHT groups are significantly different at $p<0.05$. n=5-8/group. Each bar represents the mean $\pm$ standard error of the mean of values obtained from individual rats within each treatment group.

Figure 13. Bright-field photomicrographs showing CRH-immunoreactive cells (A,B,C) and AVP-immunoreactive cells (D) within the paraventricular nucleus of intact (A), gonadectomized (B,D), and gonadectomized, dihydrotestosterone- (DHT) treated (C) male F344 rats. Animals were gonadectomized 3 weeks prior to sacrifice. DHT was administered continuously from the time of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate subcutaneously implanted in each animal's nape. The third ventricle is on the right. Bar = 100 μm.

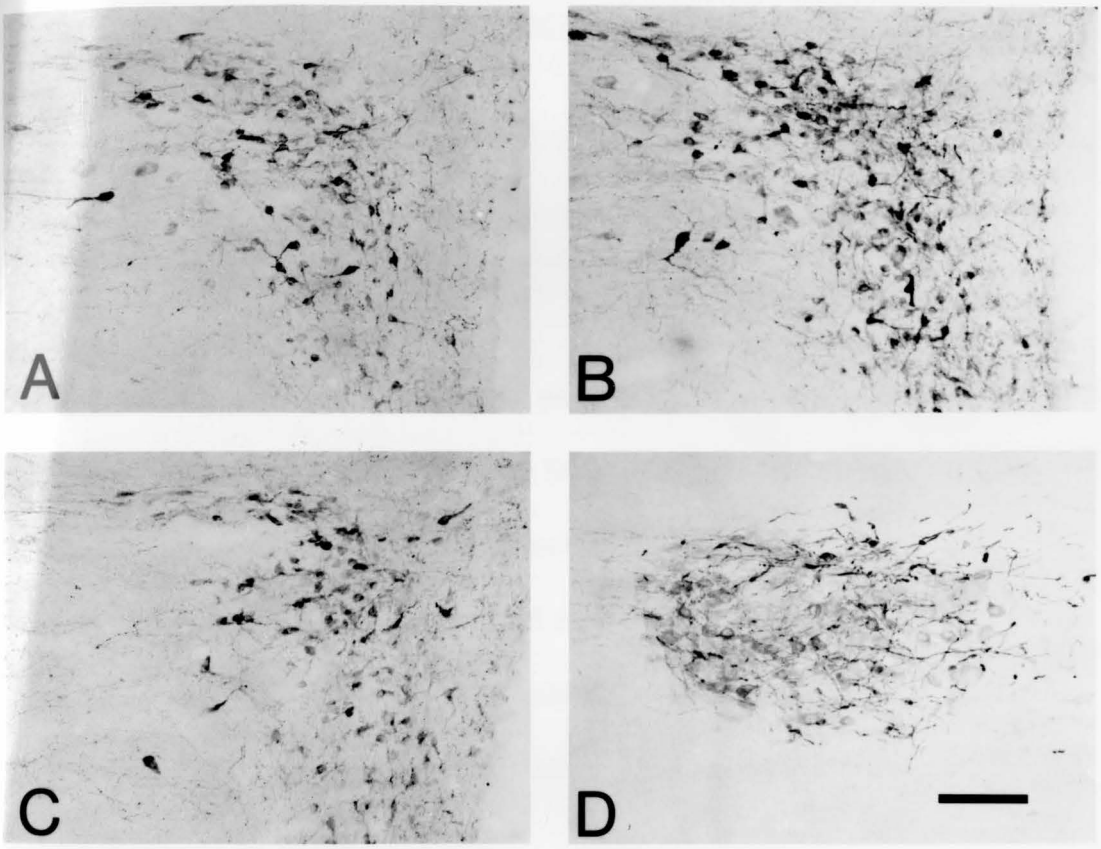

A
B
C
D

TABLE 3. Plasma testosterone and dihydrotestosterone (DHT) levels in intact, gonadectomized, and gonadectomized, DHT-treated male F344 rats determined by radioimmunoassay.[a,b]

Treatment[c]	Testosterone (n)[d,e]	DHT (n)[d,e]
INT	1.267 ± .167 (10)	.255 ± .065 (7)
GDX	ND (9)	ND (5)
GDX + DHT	ND (9)	1.538 ± .123 (6)*

[a]Animals were gonadectomized 3 weeks prior to sacrifice. DHT was administered at the time of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate implanted under the skin of each animal's back.

[b]Plasma hormone levels were determined in animals used for both CRH radioimmunoassay and immunocytochemistry.

[c]INT, intact; GDX, gonadectomized; GDX + DHT, gonadectomized, DHT-treated

[d]values expressed in ng/ml

[e]n, number of animals per group

ND designates values too low to detect. The limits of detectability were 0.040 ng/ml for testosterone and 0.145 ng/ml for DHT.

* Plasma DHT levels of GDX + DHT animals were significantly ($p < .01$) higher than in intact animals.

GDX, and GDX + DHT rats are shown in Figure 13.

Discussion

These data demonstrate that long term castration increases hypothalamic CRH concentration. This increase in CRH in the gonadectomized male rat correlates with the increased CORT and ACTH responses to physical and psychological stressors previously observed in the gonadectomized male rat (Handa *et al.*, 1994; Viau and Meaney, 1991b) and suggests that this pool of CRH is readily releasable. Although a previous study suggested that prepuberal gonadectomy of male rats did not affect hypothalamic content of corticotropin-releasing bioactivity (Coyne and Kitay, 1971), our data are consistent with a more recent study showing increases in hypothalamic CRH peptide levels following long term castration (Almeida *et al.*, 1992). Also consistent with this study, we report here that greater than ten days is necessary to see an effect of castration on hypothalamic CRH content. This effect is most probably due to a release of inhibition by androgens since we have demonstrated that androgen replacement restores hypothalamic CRH concentration to that of the intact animal. This inhibition appears to be an androgen receptor-mediated event as the non-aromatizable androgen, DHT, was effective in suppressing castration-induced increases in hypothalamic CRH concentration. Although it may appear that the levels of plasma DHT in the DHT-treated castrates were supra-physiologic, it was our intention to replace the total androgen (testosterone and DHT) lost due to the gonadectomy. It should also be noted that our values for hypothalamic CRH content

are in the range of those previously reported (Almeida *et al.*, 1992).

We have demonstrated that CRH-IR cell numbers in the PVN of gonadectomized male rats are greater than in gonadectomized rats receiving replacement DHT titers. This suggests that in the absence of androgens CRH synthesis or storage may be increased such that more cells can be detected immunocytochemically. Alternatively, a subpopulation of cells within the PVN may begin to synthesize CRH. It should be noted that the effect of androgens on CRH-IR cell numbers in the PVN reported here was observed in animals that were not pre-treated with colchicine. The accumulation of CRH that results from treatment with colchicine could mask the effect of gonadal steroids on CRH immunoreactivity.

Whether androgen-induced changes in CRH-IR cell numbers and hypothalamic CRH concentration occur as a result of changes in synthesis or degradation of CRH is unknown. However, recent studies showing that CRH mRNA levels in the PVN are not altered by short- or long-term castration (Almeida *et al.*, 1992) suggest that CRH synthesis may not be responsible for the increases in CRH content and CRH-IR cell numbers observed in the present study.

In the brain, testosterone can act as either an androgen or an estrogen depending upon its intracellular fate (for rev. see McEwen and Parsons, 1982). After testosterone has passed through the neuronal membrane, it can bind directly to androgen receptor or it can be reduced by the intracellular enzyme, 5α-reductase, to DHT which also binds with high affinity to androgen receptor. Alternatively, if the aromatase enzyme is present within the cell, testosterone can be converted to 17β-

estradiol (Weisz and Gibbs, 1974; Naftolin *et al.*, 1975). This 17β-estradiol can then bind to estrogen receptors and will thus influence cellular function in an alternate manner. The effect of androgens on hypothalamic CRH concentration and CRH-IR cell numbers in the PVN appears to be an androgen receptor-mediated phenomenon as the non-aromatizable androgen, DHT, was effective in reversing the effects of castration. It is uncertain, however, whether androgens are acting directly on CRH containing neurons in the PVN. Although both [^{3}H]-DHT uptake (Sar and Stumpf, 1977) and androgen receptor mRNA (Simerly *et al.*, 1990) have been detected in the PVN, androgen receptor mRNA may not be localized in the parvocellular region of the PVN where CRH containing neurons are found (Simerly *et al.*, 1990). Consequently, androgens may exert an effect on the PVN transsynaptically as androgen receptor (Sar and Stumpf, 1977; Sheridan, 1979; Handa *et al.*, 1987) and androgen receptor mRNA (Simerly *et al.*, 1990) are found within brain regions which project (directly or indirectly) to the PVN such as the BNST (Swanson and Cowan, 1979; Silverman *et al.*, 1981) and hippocampus (for rev. see Jacobson and Sapolsky, 1991). Furthermore, the effect of androgens on CRH-IR cell numbers appears to be region-specific as no differences were seen among the treatment groups in either the BNST or the CeA. This region-specificity of androgen action might better be explained by a transsynaptic mechanism such as that hypothesized for steroidal regulation of gonadotropin-releasing hormone (Petraglia *et al.*, 1984; Bhanot and Wilkinson, 1985; Gabriel *et al.*, 1986; Wehrenberg *et al.*, 1989; Kalra *et al.*, 1990; Sahu *et al.*, 1990; Huang and Harlan, 1993).

Arginine vasopressin (AVP) is present in both intra- and extrahypothalamic sites (Buijs, 1978). AVP has been colocalized with CRH in hypothalamic neurons and potentiates the effect of CRH on ACTH secretion (Gillies *et al.*, 1982; Antoni *et al.*, 1983; Rivier *et al.*, 1984b; Sawchenko *et al.*, 1984; Roth *et al.*, 1982). The density of AVP fibers originating in the BNST (DeVries and Buijs, 1983) and innervating the lateral septum and lateral habenular nucleus is higher in males than in females (DeVries *et al.*, 1981). Furthermore, this sex difference is dependent on the neonatal presence of testosterone (DeVries *et al.*, 1983). In the adult animal, androgens appear to maintain this sex difference since gonadectomy of male rats leads to a disappearance of AVP-IR cell bodies in the BNST (DeVries *et al.*, 1985; Miller *et al.*, 1992) and medial amygdala without affecting AVP-IR cell bodies in the PVN (DeVries *et al.*, 1985). Our studies also demonstrate that AVP-IR cell numbers in the PVN were not influenced by the androgen status of the animal. While it is clear that androgens can regulate AVP expression in some brain regions, changes in the AVP system would not explain changes noted in the CORT and ACTH response to stress noted in the castrated male rat (Handa *et al.*, 1994; Viau and Meaney, 1991b).

In summary, the results of the present study demonstrate that castration increases and androgens decrease hypothalamic CRH content and CRH-IR cell numbers in the PVN. These parameters can be influenced by synthesis in the soma, secretion from the terminals, and degradation of the neuropeptide. It is unknown at this point which of these factors is altered by androgens. Additionally, while our data indicate that androgen's influence on the HPA axis occurs, at least in part, at the level

of the hypothalamus, it should not be inferred that androgens act directly on the hypothalamus. The PVN is a highly integrated and regulated cell group (for rev. see Swanson and Sawchenko, 1983), and many possible target sites exist at which gonadal steroids could act to ultimately regulate CRH cells within the PVN. There may exist a complex pathway by which the circulating gonadal hormone milieu influences adaptive physiological responses to environmental perturbations. It is clear that an extensive interrelationship exists between the HPA and the hypothalamo-pituitary-gonadal axes.

CHAPTER IV

CIRCULATING ANDROGENS DO NOT ALTER CORTICOTROPIN-RELEASING HORMONE mRNA LEVELS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

Summary

Previous studies demonstrate that long term castration increases hypothalamic corticotropin-releasing hormone (CRH) content and CRH immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) of male rats by removal of an androgen-dependent repression. To characterize the effect of androgens on hypothalamic CRH, we examined the effect of gonadectomy and androgen treatment on CRH mRNA levels in the PVN of male rats. Three month-old, male Fischer 344 (F344) rats were bilaterally gonadectomized under ether anesthesia. Gonadectomized animals received either the non-aromatizable androgen, dihydrotestosterone (DHT), using a Silastic capsule containing crystalline DHT propionate subcutaneously implanted in each animal's back (GDX+DHT) or a sham incision (GDX). Control animals remained intact. For positive controls, male rats were adrenalectomized (ADX) and sacrificed seven days after surgery. Corticotropin-releasing hormone mRNA was examined by *in situ* hybridization histochemistry using a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA.

Arginine vasopressin (AVP) mRNA was similarly evaluated using a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 286-315 of prepro-AVP mRNA. Image analysis of hybridization density in the PVN showed no significant differences in CRH mRNA or AVP mRNA between intact, GDX, and GDX+DHT animals sacrificed 3 weeks after surgery. Similarly, the numbers of CRH mRNA-labelled cells in the dorsal medial parvocellular, dorsal parvocellular, and ventral medial parvocellular subdivisions of the PVN and the numbers of AVP mRNA-labelled cells in magnocellular and parvocellular subdivisions of the PVN were not altered 3 weeks after gonadectomy. In addition, there were no significant differences in CRH mRNA hybridization in the PVN or in the numbers of CRH mRNA-labelled parvocellular neurons among intact, GDX, and GDX, DHT-treated rats sacrificed 10 days after surgery. These data suggest that changes in hypothalamic CRH content and CRH immunoreactivity in the PVN after gonadectomy cannot be explained by changes in CRH gene expression.

Introduction

Activation of the hypothalamo-pituitary adrenal (HPA) axis is a major component of an organism's adaptive biological reaction to stressful stimuli. In response to stress, neurosecretory cells in the parvocellular portion of the paraventricular nucleus of the hypothalamus (PVN) synthesize and secrete corticotropin-releasing hormone (CRH) into the hypohysial portal vasculature. CRH serves as the major physiological stimulus promoting the secretion of

adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH subsequently causes the adrenal cortex to secrete corticosterone (CORT) into the peripheral circulation. During stress, corticosterone influences several physiological parameters including glucose mobilization, inflammation, and water resorption.

The interactions that take place between the HPA axis and the hypothalamo-pituitary-gonadal axis have been widely studied. Hypothalamo-pituitary-adrenal axis hormones inhibit gonadotropin secretion and reproductive behavior (Rivier and Vale, 1984; Rivier and Vale, 1985b; Rivier *et al.*, 1986; Kamel and Kubajak, 1987; Plas-Rose and Aron, 1981; Sirinathsinghji *et al.*, 1983; Armstrong, 1986; Sirinathsinghji *et al.*, 1986). Conversely, gonadal hormones affect HPA activity. In rats, females have higher circulating CORT levels and higher CORT responses to stress than males (Kitay, 1961). Estrogen contributes to this sex difference by increasing HPA activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992). In contrast, circulating androgens may inhibit HPA activity (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994).

Circulating androgens appear to regulate the HPA axis, at least in part, at the level of the hypothalamus. Previous studies have demonstrated that hypothalamic CRH content is increased following gonadectomy of male rats (Bingaman *et al.*, 1994). In addition, treatment of gonadectomized rats with dihydrotestosterone (DHT) normalizes hypothalamic CRH content to that of intact animals. This is consistent with studies which indicate that the ACTH and corticosterone responses to a physical or psychological stressor are increased in gonadectomized male rats (Handa *et al.*,

1994; Viau and Meaney, 1991b).

Several mechanisms could explain the regulation of hypothalamic CRH by androgens. Possibilities include an increased synthesis or half-life of CRH or a decrease in axoplasmic transport and/or secretion following gonadectomy. This experiment was performed to determine whether the influence of androgens on CRH content and CRH immunoreactivity in the hypothalamus is a consequence of changes in CRH gene expression. Corticotropin-releasing hormone mRNA levels in the PVN of intact, gonadectomized, and gonadectomized, DHT-treated male rats were evaluated using *in situ* hybridization histochemistry (ISHH). Arginine vasopressin (AVP) mRNA levels in the PVN of these animals were similarly evaluated due to AVP's role as a corticotropin-releasing factor.

Changes in CRH content may reflect concurrent changes in CRH mRNA. Alternatively, in the castrate CRH synthesis may increase briefly and then return to steady state once the larger CRH pool is established. If the latter case is true, the transient increase in mRNA levels would be expected to precede changes in protein content. Since hypothalamic CRH content begins to increase 10 days after gonadectomy (Bingaman *et al.*, 1994) with a significant increase by 21 days after castration CRH mRNA levels were evaluated at 10 or 21 days following surgery.

Materials and Methods

Animals and Surgery

Three month-old, male Fischer 344 rats (F344, Harlan/Sprague Dawley,

Indianapolis, IN) were maintained on a 12:12 light:dark schedule with lights on at 0700h. Animals were housed in temperature controlled rooms. Food and water were available ad libitum. Rats were bilaterally gonadectomized under ether anesthesia. Gonadectomized animals received either hormone replacement using a 2.5 cm Silastic capsule (0.07" I.D., 0.125" O.D.; Dow Corning, Midland, MI) containing crystalline DHT propionate subcutaneously implanted in each animal's back (GDX+DHT) or a sham incision (GDX). Control animals were left intact (INT). Animals were sacrificed either 10 or 21 days after surgery between 0900 and 1100 h. For positive controls, three month-old male F344 rats were adrenalectomized under ether anesthesia (ADX) and sacrificed seven days after surgery. All animals were sacrificed by decapitation immediately following removal from their home cage. Trunk blood was collected into tubes containing 300 μl 0.3 M ethylenediaminetetraacetic acid (EDTA). Plasma was frozen (-80°C) until assayed for testosterone and DHT.

Tissue Preparation

Brains were immediately removed, blocked, and frozen in isopentane (-20°C) for 30 seconds and then placed on dry ice. Brains were stored at -80°C until 18 μm-thick frontal sections were cut on a cryostat. Sections were cut to include the PVN. Sections were stored dessicated and frozen (-80°C) until use.

Preparation of Labelled Probes

Oligonucleotide probes complementary to nucleotides 496-543 of rat CRH mRNA and nucleotides 286-315 of prepro-AVP mRNA were radiolabelled by poly-A tailing in the presence of ^{35}S-ATP (NEN; Boston, MA) using terminal deoxynucleotidyl transferase (Boehringer Mannheim; Indianapolis, IN). The specific activity of the CRH oligonucleotide probe was approximately 3.20×10^{18} dpm/mol. The specific activity of the AVP oligonucleotide probe was approximately 3.43×10^{18} dpm/mol.

In Situ Hybridization

Every fourth section was used for evaluation of CRH mRNA levels. Sections were processed for ISHH using the ^{35}S-labelled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA. Adjacent sections from rats treated for 21 days were analyzed using the ^{35}S-labelled oligonucleotide probe complementary to nucleotides 286-315 of prepro-AVP mRNA. Sections were warmed to room temperature, fixed in 4% formaldehyde, acetylated with 0.25% acetic anhydride in 0.1M triethanolamine/0.9% NaCl before dehydrating and delipidating in ethanols and chloroform, respectively, and subsequent air drying. Radiolabelled probe was added at a concentration of approximately 1.5×10^6 cpm/slide in 100 μl of hybridization buffer (600mM NaCl, 10mM Tris-HCl, 0.02% Denhart's, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mg/ml denatured Herring testis DNA, 0.5 mg/ml total yeast RNA, 0.05 mg/ml yeast tRNA, 10% dextran sulfate, 0.05%

sodium thiosulfate, 50 mM DTT, 0.05% sodium dodecylsulfate (SDS), 50% formamide). Hybridization was performed overnight (16-20 hours) at 37°C. Nonspecific binding was removed by washing in 2xSSC at room temperature (1xSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 8.0), 2xSSC/50% formamide at 40°C, and 1x SSC at room temperature. Following dehydration in ethanols and drying slides were apposed to Hyperfilm β-max (Amersham; Arlington Heights, IL). Autoradiograms were analyzed by image analysis of hybridization density. Hybridized regions of the PVN were outlined using NIH Image software. The density within was measured by comparison to a standard curve created by a slide of ^{14}C standards (Amersham, Arlington Heights, IL) which were converted to their ^{35}S equivalents expressed as dpm/mg protein. (^{14}C standards are used rather than ^{35}S due to the long half-life of ^{14}C.) Next, sections were dipped in Kodak NTB-3 nuclear emulsion and exposed in a light-tight box for 3 1/2 weeks (CRH) or 3 days (AVP). Sections were then stained with cresyl violet and labelled cells were counted under darkfield microscopy. A cresyl violet-stained nucleus surrounded by exposed silver grains was considered to be a radiolabelled cell. CRH mRNA expressing cells were counted in three separate areas of the PVN (dorsal medial parvocellular, ventral medial parvocellular, and dorsal parvocellular) as described by Swanson and Simmons (1989). These areas are those in which CRH neurons are concentrated and were quantified separately so that changes in any one functional division would not be masked by lack of changes in other regions. Similarly, cells radiolabelled with the AVP mRNA probe were counted separating cells observed in the magnocellular and

parvocellular subdivisions. Cell counts were corrected for double-counting errors (Abercrombie, 1946).

Steroid Radioimmunoassays

Plasma testosterone levels were evaluated by radioimmunoassay (RIA) using a ^{125}I-testosterone kit (ICN Biomedicals, Inc., Casa Mesa, CA). The standard curve ranged from 5 to 500 pg/tube. Bound and free steroid were separated using a goat anti-rabbit gamma-globulin. The RIA sensitivity limit was 1.98 pg/tube. The intra-assay coefficient of variance was 9.5%. All values were analyzed in one assay to avoid inter-assay variation.

For *plasma DHT* determination, steroids were extracted from samples using hexane:ethyl acetate (3:2) and separated on microcelite columns (ICN Biomedicals, Inc.) using an isooctane-ethyl acetate system. Dihydrotestosterone was eluted from the column with 5% ethyl acetate in isooctane. Recovery of DHT was evaluated by adding a known amount of ^{3}H-DHT to the plasma sample prior to placement on the column. Recovery of DHT was 80 %. Plasma DHT was determined by RIA using a ^{3}H-dihydrotestosterone RIA kit (ICN Biomedicals, Inc.). The standard curve ranged from 10 to 500 pg/tube. Bound and free steroid were separated using dextran-coated charcoal. The RIA sensitivity limit was 30.3 pg/tube. The intra-assay coefficient of variance was 10.9%. All values were analyzed in one assay to avoid inter-assay variation.

Statistics

The data are represented as the group means and the standard errors of the mean (S.E.M.). *In situ* hybridization histochemistry data were analyzed by one-way analysis of variance, and Newman-Keuls' test was used for post hoc analysis (Winer, 1977). Plasma DHT levels were analyzed by a two-tailed t-test (Winer, 1977). A p value of < 0.05 was considered significant.

Results

Plasma testosterone and DHT concentrations are shown in Table 4. Gonadectomy reduced circulating androgens, and hormone treatment increased DHT titers. It may appear as though the levels of plasma DHT in the DHT-treated castrates were supra-physiologic. However, it was our intention to replace the total androgen (testosterone and DHT) lost due to gonadectomy.

Figure 14 shows the hybridization density of a ^{35}S-labelled oligonucleotide probe for CRH mRNA in the PVN of INT, GDX, GDX+DHT, and ADX animals. Hybridization of the ^{35}S-labelled probe to CRH mRNA was significantly increased ($p<.05$) in the PVN of adrenalectomized animals compared to INT animals. However, there were no significant differences in hybridization density in the PVN between INT, GDX, and GDX+DHT animals 21 days after surgery. Figure 15

Table 4. Plasma testosterone and dihydrotestosterone (DHT) levels in intact, gonadectomized, and gonadectomized, DHT-treated male F344 rats determined by radioimmunoassay.[a]

Treatment[b]	Testosterone (n)[c,d]	DHT (n)[c,d]
INT	0.65 ± 0.14 (11)	0.24 ± 0.05 (7)
GDX	ND (11)	ND (9)
GDX + DHT	ND (9)	1.50 ± 0.16 (6)*

[a]Animals were gonadectomized 10 days or 3 weeks prior to sacrifice. DHT was administered at the time of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate implanted under the skin of each animal's back.

[b]INT, intact; GDX, gonadectomized; GDX + DHT, gonadectomized, DHT-treated

[c]values expressed in ng/ml

[d]n, number of animals per group

ND designates values too low to detect. The limits of detectability were .040 ng/ml for testosterone and 0.145 ng/ml for DHT.

* Plasma DHT levels of GDX + DHT animals were significantly ($p < .01$) higher than in intact animals.

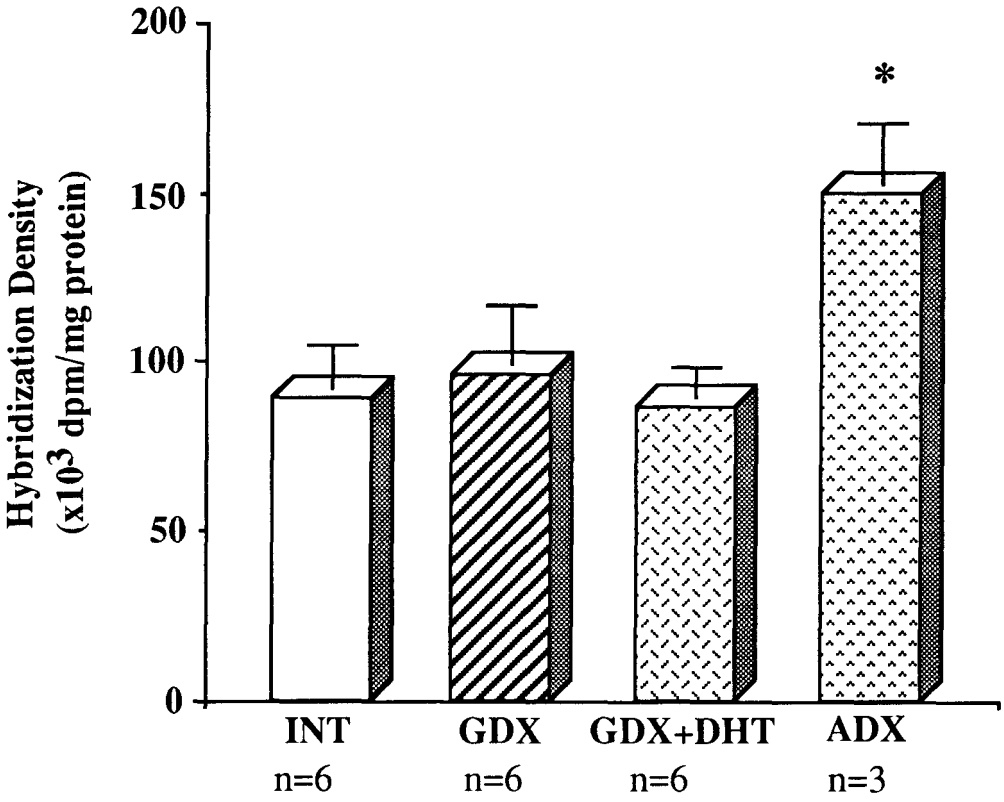


Figure 14. Hybridization density of a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA in the paraventricular nucleus (PVN) of male F344 rats. Animals were either intact (INT), were bilaterally gonadectomized under ether anesthesia, or were adrenalectomized (ADX). Gonadectomized animals received either DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT) or a sham operation (GDX). GDX and GDX+DHT animals were sacrificed by decapitation 21 days after surgery. ADX animals were sacrificed 7 days after surgery. * designates groups which are significantly different from intact group ($p < .05$).

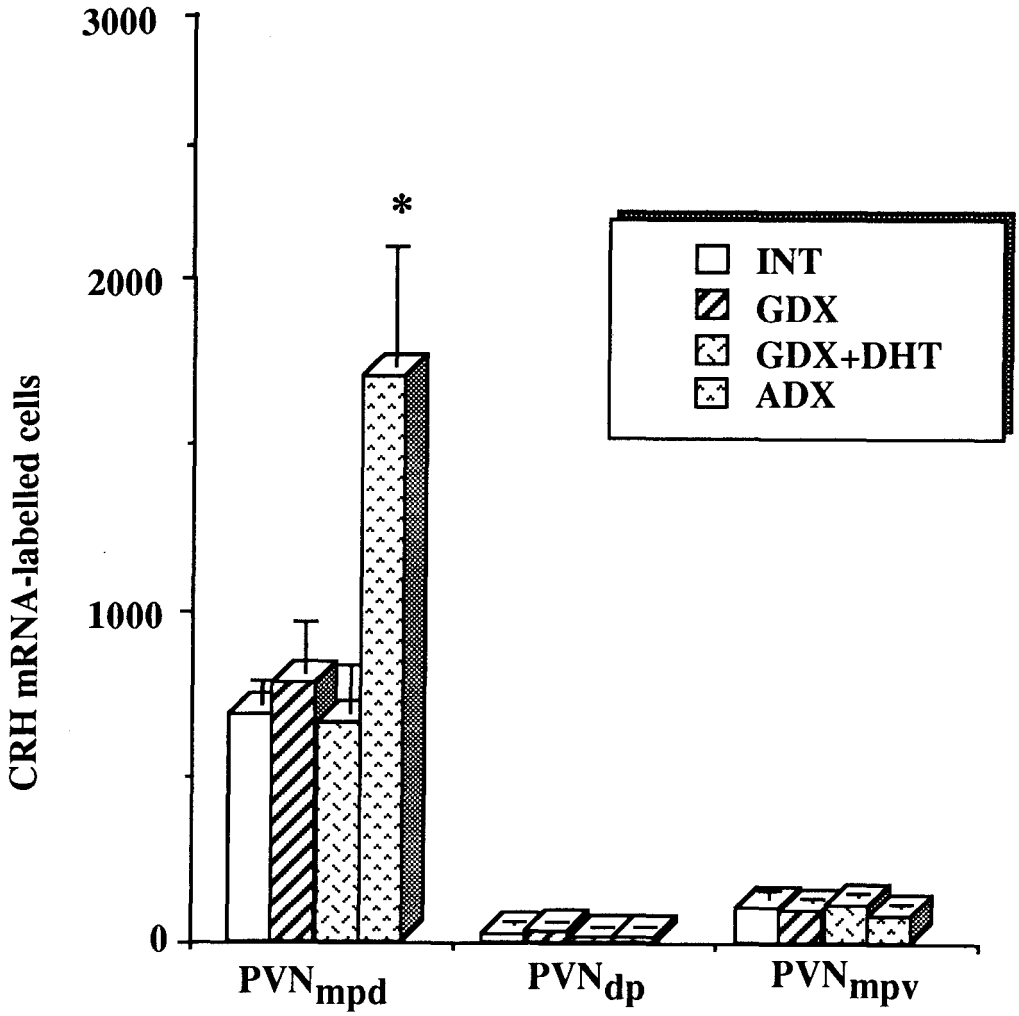
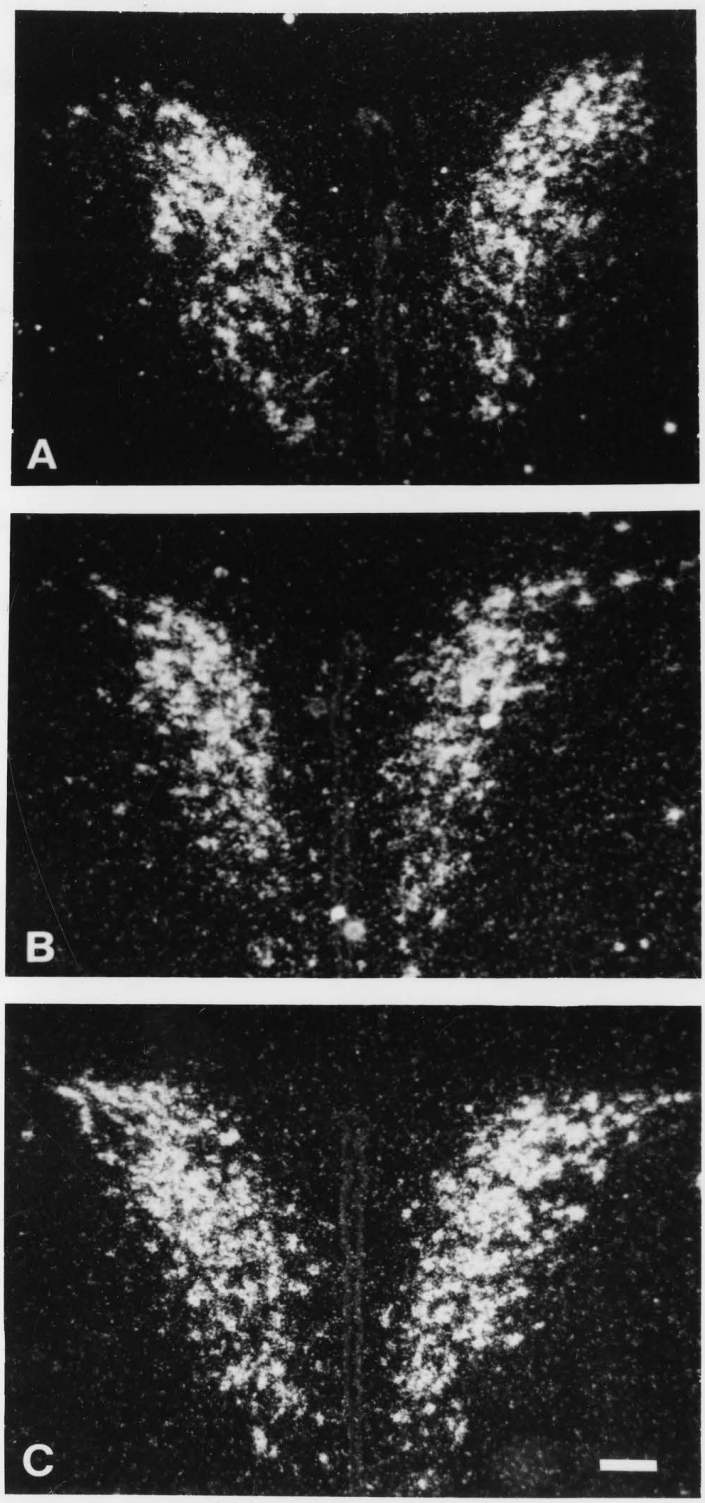


Figure 15. Numbers of cells in the dorsal medial parvocellular (PVN_{mpd}), dorsal parvocellular (PVN_{dp}), and ventral medial parvocellular (PVN_{mpv}) subdivisions of the hypothalamic paraventricular nucleus radiolabelled using a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA. Animals were either intact (INT), were bilaterally gonadectomized under ether anesthesia, or were adrenalectomized (ADX). Gonadectomized animals received either DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT) or a sham operation (GDX). GDX and GDX+DHT animals were sacrificed by decapitation 21 days after surgery. ADX animals were sacrificed 7 days after surgery. * designates groups which are significantly different from intact group ($p < .01$). n=3-6.

Figure 16. Darkfield photomicrographs demonstrating CRH mRNA hybridization over the paraventricular nucleus of the hypothalamus of intact (A), gonadectomized (B), and adrenalectomized (C) male rats. Animals were gonadectomized 3 weeks prior to sacrifice. Adrenalectomized animals were sacrificed 7 days after surgery. Bar = 100 μm.


A
B
C

shows the number of cells positively labelled for CRH mRNA in three regions of the PVN of INT, GDX, GDX+DHT, and ADX male rats. There were significantly more ($p < .01$) CRH mRNA-labeled cells in the dorsal medial parvocellular subdivision of the PVN (PVN_{mpd}) in ADX animals compared to intact rats. However, in the PVN_{mpd} the numbers of CRH mRNA-labelled cells in INT, GDX, andGDX+DHT animals were not significantly different. In addition, the numbers of CRH mRNA-labelled cells in the dorsal parvocellular (PVN_{dp}) and ventral medial parvocellular (PVN_{mpv}) subdivisions did not significantly differ among the treatment groups. Photomicrographs of representative frontal sections demonstrating CRH mRNA expressing cells in the PVN of INT, GDX, and ADX male rats are shown in Figure 16.

The hybridization density of a ^{35}S-labelled oligonucleotide probe for AVP mRNA in the PVN of INT, GDX, GDX+DHT, and ADX animals is shown in Figure 17. There were no significant differences in hybridization density in the PVN among the treatment groups. However, the area hybridized was significantly greater ($p < .05$) in ADX animals compared to intact animals (INT, 1.03 ± 0.07 mm^2; ADX, 1.88 ± 0.27 mm^2). The numbers of cells positively labelled for AVP mRNA in the magnocellular (PVN_M) and parvocellular (PVN_p) subdivisions of the PVN of INT, GDX, GDX+DHT, and ADX male rats are shown in Figure 18. There were significantly ($p < .01$) more radiolabelled cells in the parvocellular, but not the magnocellular, subdivision of the PVN in ADX animals compared to INT animals. However, there were no significant differences in numbers of radiolabelled cells

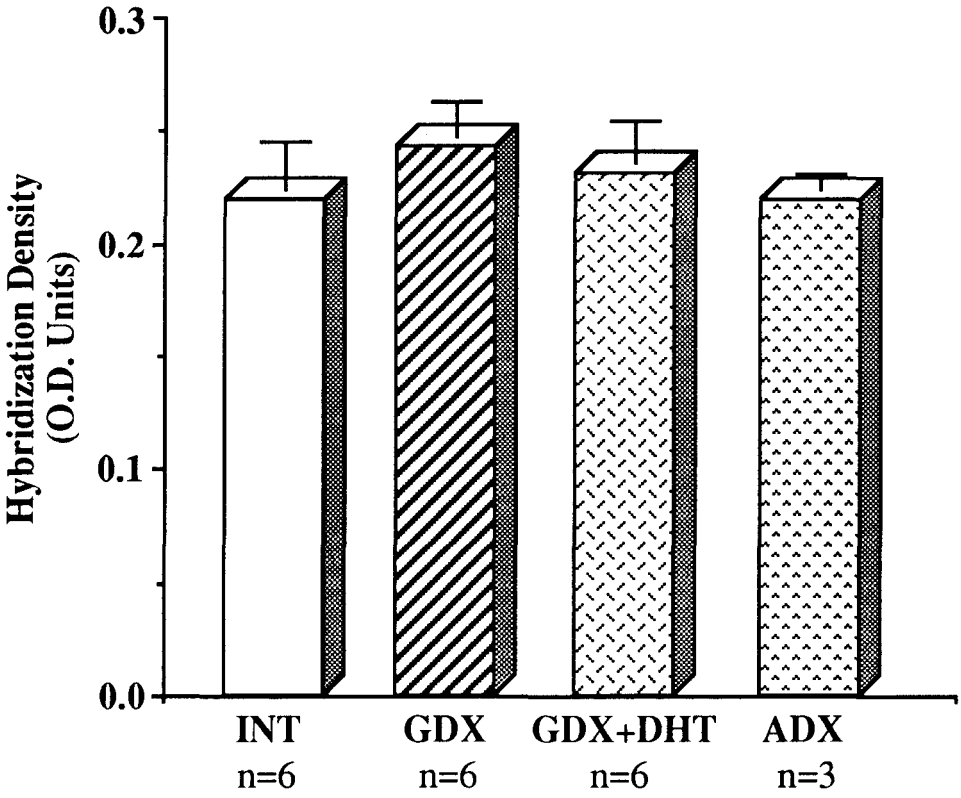


Figure 17. Hybridization density of a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 286-315 of prepro-AVP mRNA in the paraventricular nucleus (PVN) of male F344 rats. Due to the short exposure time for sections hybridized with the probe for prepro-AVP mRNA, a standard curve could not be generated. Therefore, hybridization density was expressed in optical density (O.D.) units. Animals were either intact (INT), bilaterally gonadectomized under ether anesthesia, or adrenalectomized (ADX). Gonadectomized animals received DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT) or a sham operation (GDX). GDX and GDX+DHT animals were sacrificed by decapitation 21 days after surgery. Adrenalectomized animals were sacrificed 7 days after surgery. It should be noted that the scale for O.D. units runs from 0 to a maximum of 1.0.

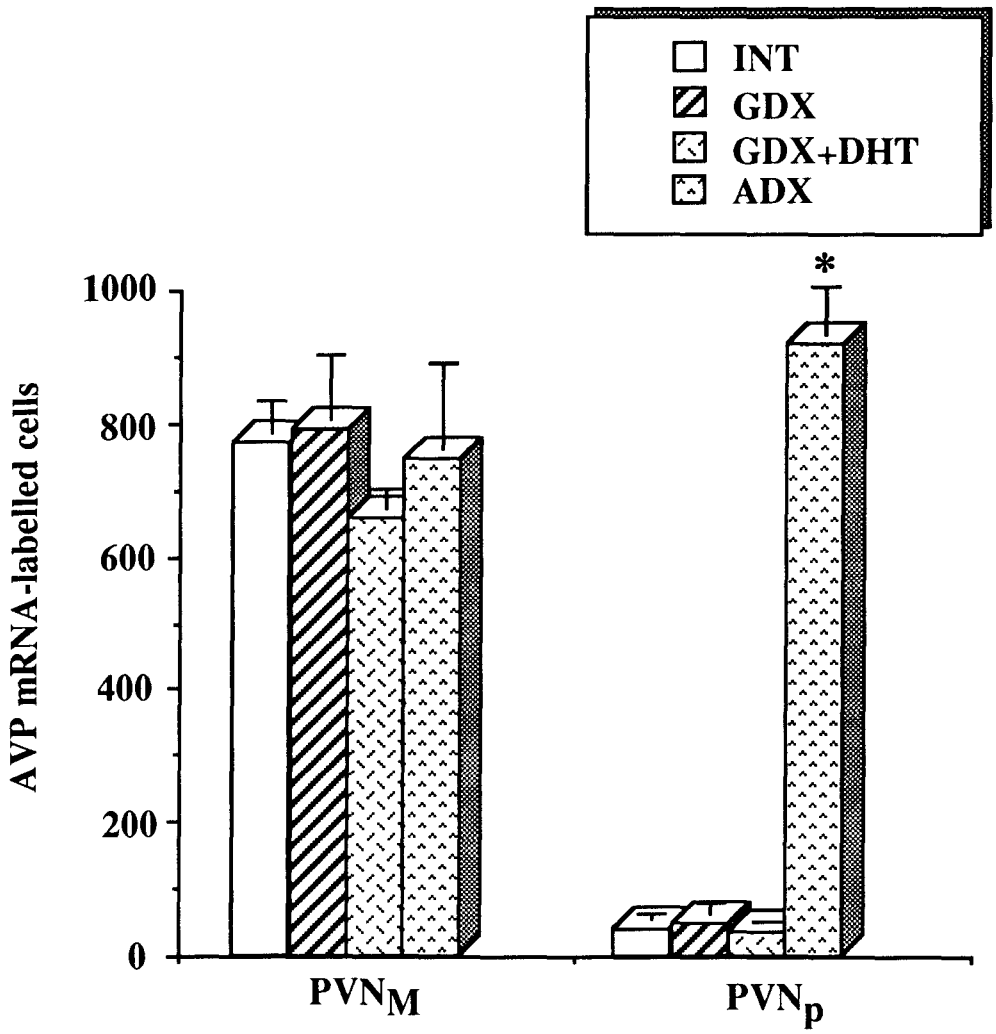
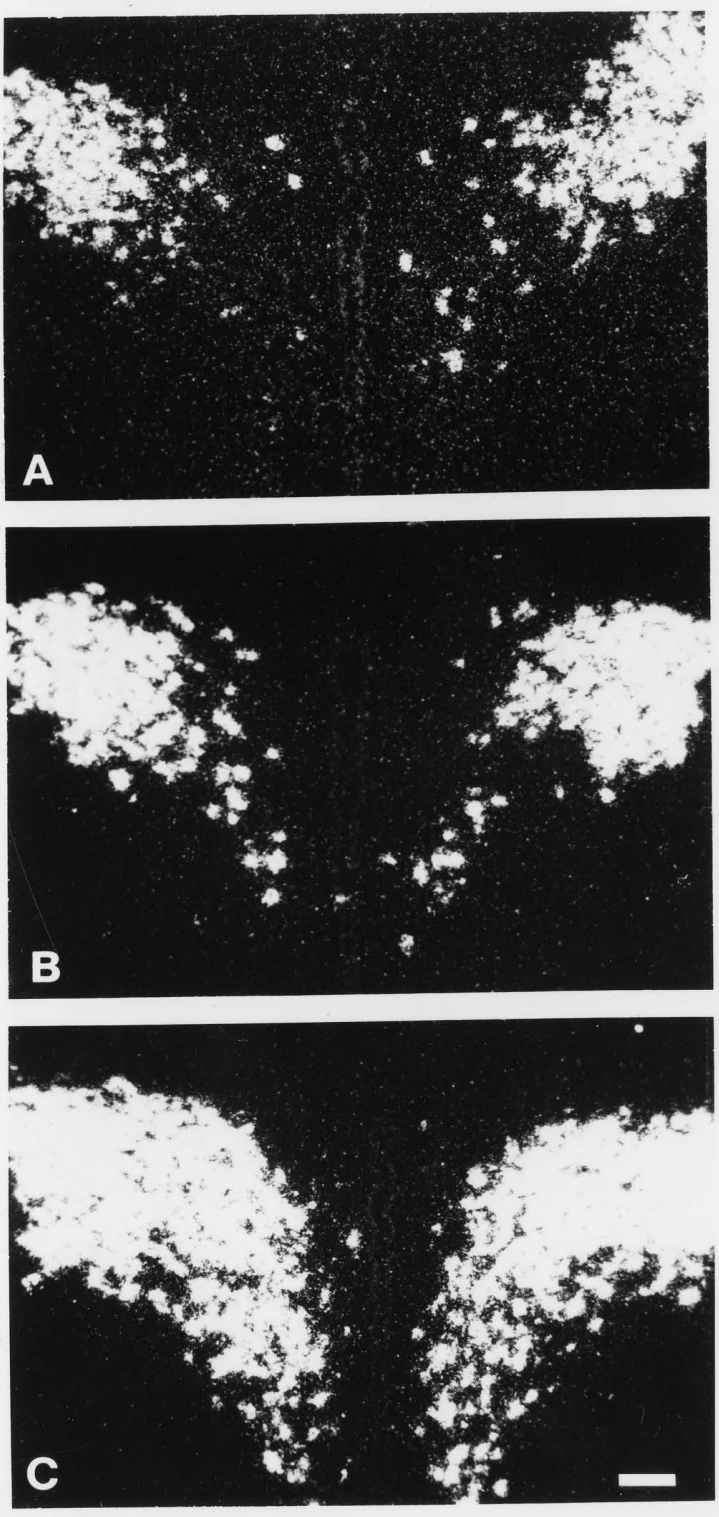


Figure 18. Numbers of cells in magnocellular (PVN_M) and parvocellular (PVN_p) subdivisions of the paraventricular nucleus of the hypothalamus radiolabelled using a ^{35}S-labelled oligonucleotide probe complimentary to nucleotides 286-315 of prepro-AVP mRNA. Male rats were either intact (INT), bilaterally gonadectomized under ether anesthesia, or adrenalectomized (ADX). Gonadectomized animals received DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT) or a sham operation (GDX). GDX and GDX+DHT animals were sacrificed by decapitation 21 days after surgery. Adrenalectomized animals were sacrificed 7 days after surgery. * designates groups which are significantly different from intacts ($p < .01$). n=3-6.

Figure 19. Darkfield photomicrographs demonstrating AVP mRNA hybridization over the paraventricular nucleus of the hypothalamus of intact (A), gonadectomized (B), and adrenalectomized (C) male rats. Animals were gonadectomized 3 weeks prior to sacrifice. Adrenalectomized animals were sacrificed 7 days after surgery. Bar = 100 μm.


A
B
C

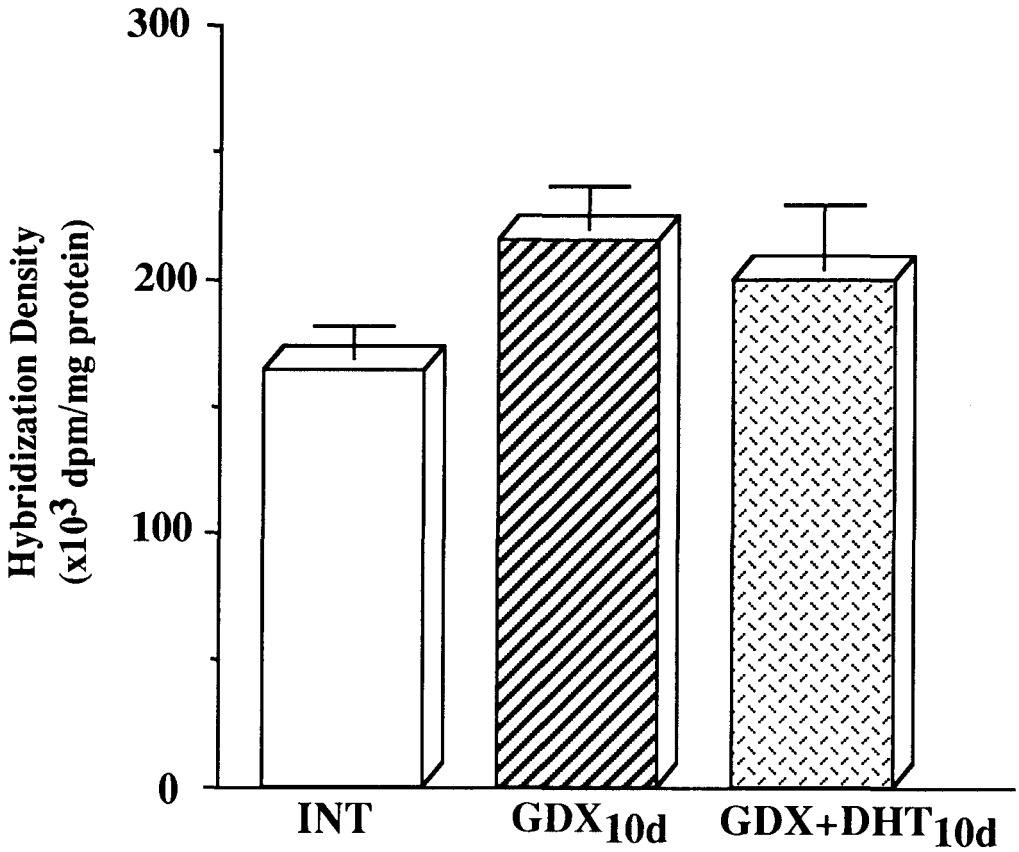


Figure 20. Hybridization density of a ^{35}S-labelled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA in the paraventricular nucleus (PVN) of male F344 rats. Animals were either intact (INT) or were bilaterally gonadectomized under ether anesthesia. Gonadectomized animals received DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT$_{10d}$) or a sham operation (GDX$_{10d}$). Animals were sacrificed by decapitation 10 days after surgery. n=6.

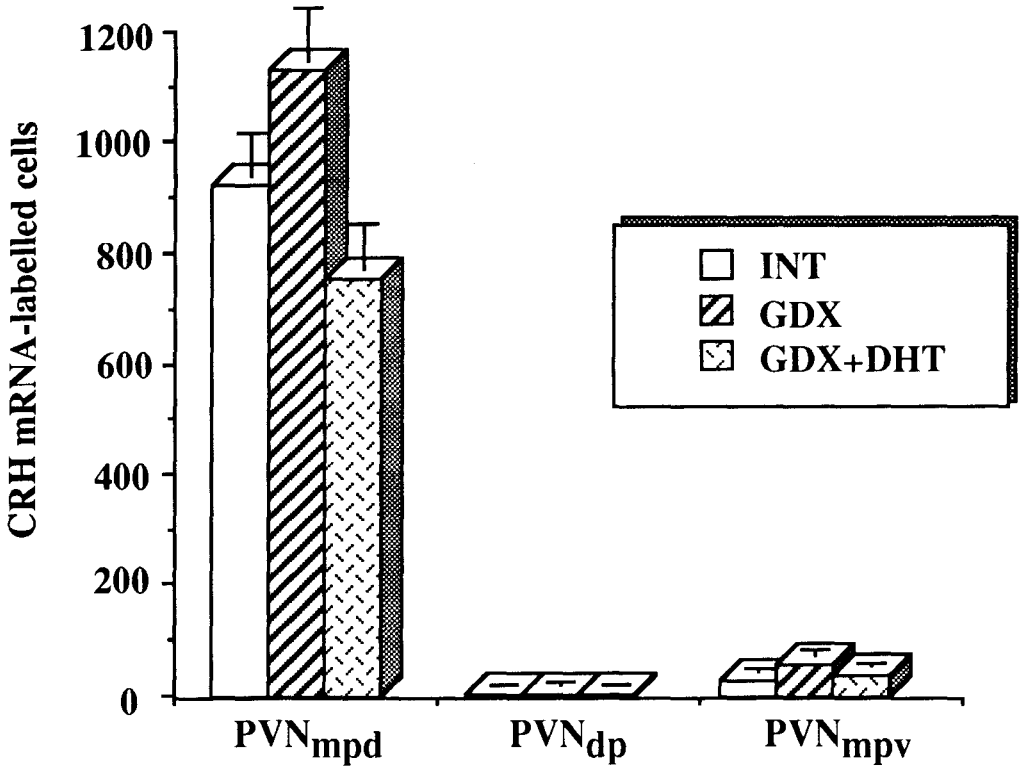


Figure 21. Numbers of cells in the dorsal medial parvocellular (PVN_{mpd}), dorsal parvocellular (PVN_{dp}), and ventral medial parvocellular (PVN_{mpv}) subdivisions of the hypothalamic paraventricular nucleus radiolabelled using a ^{35}S-labeled oligonucleotide probe complementary to nucleotides 496-543 of rat CRH mRNA. Animals were either intact (INT) or were bilaterally gonadectomized under ether anesthesia. Gonadectomized animals received DHT using a 2.5 cm Silastic capsule subcutaneously implanted in each animal's back (GDX+DHT) or a sham operation (GDX). Animals were sacrificed by decapitation 10 days after surgery. n=4-6.

among INT, GDX, and GDX+DHT rats. Photomicrographs of representative frontal sections showing AVP mRNA expressing cells in the PVN of INT, GDX, and ADX male rats are shown in Figure 19.

Figure 20 shows the hybridization density of the ^{35}S-labelled oligonucleotide probe for CRH mRNA in the PVN of intact male rats (INT), male rats that have been castrated for 10 days (GDX_{10d}), and rats that have been castrated and DHT-treated for 10 days ($GDX+DHT_{10d}$). There were no significant differences in hybridizationdensity among these treatment groups. The numbers of cells positively labelled for CRH mRNA in three subdivisions of the PVN (PVN_{mpd}, PVN_{dp}, and PVN_{mpv}) of INT, GDX_{10d}, and $GDX+DHT_{10d}$ are shown in Figure 21. The number of radiolabelled cells did not significantly differ among the treatment groups in any of the three subdivisions.

Discussion

Previous studies suggest that circulating androgens inhibit the activity of the HPA axis (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Viau and Meaney, 1991b; Handa *et al.*, 1994). For example, the ACTH and corticosterone responses to footshock or novel environment stress are increased in male rats three weeks after gonadectomy (Handa et al., 1994). Furthermore, treatment of gonadectomized males with testosterone or the non-aromatizable androgen, DHT, normalizes the ACTH and corticosterone responses to these stressors. The regulation of the HPA axis by circulating androgens appears to occur, at least in part, at the level of the

hypothalamus. Hypothalamic CRH content is increased three weeks after castration in male rats (Bingaman, *et al.*, 1994).

The results of the present study indicate that CRH mRNA levels and the number of CRH mRNA expressing cells in parvocellular PVN neurons are not altered three weeks after castration. This is consistent with a previous study which suggests that 7 or 28 days of gonadectomy does not alter CRH mRNA in the PVN (Almeida *et al.*, 1992). Thus, the changes observed in hypothalamic CRH content and CRH-immunoreactive cell numbers in the PVN after gonadectomy of male rats (Bingaman *et al.*, 1994) cannot be explained by concurrent changes in CRH mRNA in the PVN. Alternatively, androgens could be suppressing the translational efficiency or decreasing the half-life of the CRH peptide.

In contrast, we observed approximately a 60% increase in CRH mRNA levels in the PVN seven days after adrenalectomy. Furthermore, adrenalectomy resulted in an increase in the number of CRH mRNA expressing cells in the PVN_{mpd}. These data are consistent with studies reporting similar increases in CRH mRNA in the PVN following adrenalectomy (Young et al., 1986a; Kovacs and Mezey, 1987; Beyer et al., 1988; Swanson and Simmons, 1989; Imaki et al., 1991). It has been reported that while corticosterone may decrease CRH gene expression in the PVN_{mpd}, high levels of corticosterone increase CRH mRNA in the PVN_{dp} and PVN_{mpv} (Swanson and Simmons, 1989). In the present study we did not observe a significant difference in the number of CRH mRNA expressing cells in the PVN_{dp} or the PVN_{mpv} in adrenalectomized animals compared to intact rats. It is possible that the levels of

circulating corticosterone in the intact rats are not sufficiently high to achieve a significant difference compared to adrenalectomized animals.

The effect of gonadectomy and androgen treatment on AVP mRNA levels in the PVN were evaluated due to AVP's role as a corticotropin-releasing factor. The data indicate that AVP mRNA levels and the number of AVP mRNA expressing cells are also not altered three weeks after gonadectomy. Adrenalectomized animals were again used as a positive control. The number of AVP mRNA expressing cells was significantly increased in parvocellular PVN neurons of adrenalectomized rats compared to intact animals. These data are consistent with previous studies (Young *et al.*, 1986b; Swanson and Simmons, 1989).

It is not surprising that circulating androgens had no effect on AVP gene expression. It was previously reported that gonadectomy of male rats did not lead to changes in AVP immunoreactivity in the PVN (DeVries *et al.*, 1985; Bingaman *et al.*, 1994). Although it is clear that androgens regulate AVP-immunoreactivity and AVP mRNA levels in the bed nucleus of the stria terminalis and medial amygdala (DeVries *et al.*, 1985; Miller *et al.*, 1992), androgens do not appear to influence AVP gene expression in the PVN.

Previously, it was reported that hypothalamic CRH content begins to rise 10 days after gonadectomy of male rats (Bingaman *et al.*, 1994). It is possible that CRH synthesis may increase briefly in the castrate and then return to steady state once the larger CRH pool is established. If this were the case, this transient increase would be expected to precede changes in protein content. However, in the present study CRH

mRNA levels in the PVN were not altered 10 days after gonadectomy. This suggests that the increase in hypothalamic CRH content following gonadectomy (Bingaman *et al.*, 1994) is not the result of a transient increase in CRH gene expression. A small increase in the number of CRH mRNA expressing cells was observed in the PVN_{mpd} 10 days after gonadectomy. This increase was not, however, statistically significant. Thus, it is possible that there is a transient increase in CRH gene expression and that this change occurs in a small subset of cells such that the effect is masked using the analysis techniques employed in this study. It is also possible that 10 days after gonadectomy is not the optimum time point to observe such a transient change in CRH gene expression.

In summary, the results of this study suggest that CRH and AVP mRNA levels in the PVN are not affected by circulating androgens. Thus, previously reported changes in hypothalamic CRH content and CRH-immunoreactive cell numbers in the PVN after gonadectomy cannot be explained by changes in CRH gene expression. It is possible that circulating gonadal androgens alter hypothalamic CRH content by altering CRH translational efficiency or peptide half-life. In addition, it should be noted that it is not known whether androgens act directly on the PVN to affect changes in CRH content. There may exist a complex pathway by which circulating androgens regulate CRH neurons and thus, the HPA axis.

CHAPTER V

LOCALIZATION OF ANDROGEN RECEPTOR-IMMUNOREACTIVITY WITHIN PEPTIDERGIC NEURONS OF THE RAT BRAIN

Summary

This study tested for the presence of androgen receptor immunoreactivity in somatostatin, galanin, vasopressin, corticotropin-releasing hormone, and oxytocin neurons in the rat forebrain. The brains of adult male Sprague/Dawley rats were fixed with 4% paraformaldehyde. Androgen receptor was visualized in coronal sections using nickel intensification of diaminobenzidine, and the neuropeptides were identified using a brown diaminobenzidine reaction product. Androgen receptor was localized to the nuclei of neurons in the septum, amygdala, cortex, hippocampus and hypothalamus. The majority of somatostatin containing neurons in the periventricular hypothalamic nucleus also contained androgen receptor. Androgen receptor was also found within galanin expressing cells in the bed nucleus of the stria terminalis and in the amygdala. Androgen receptor was not observed in corticotropin-releasing hormone, vasopressin, or oxytocin neurons in any area examined. The data suggest that androgens may be capable of directly regulating somatostatin expressing neurons of the periventricular nucleus of the hypothalamus and galanin containing neurons of the bed nucleus of the stria terminalis and amygdala.

Introduction

The regulation of cellular processes by androgens requires the presence of the androgen receptor (AR) which acts as a ligand-responsive transcription factor. The distribution of AR in the brains of several species has been described previously (Handa *et al.*, 1987; Sar and Stumpf, 1975). In the rat, AR is localized throughout many forebrain regions including the septum, amygdala, cortex, hippocampus, and hypothalamus. This distribution overlaps that of many neuropeptide transmitters and hormones.

Several recent studies have examined the regulation of neuropeptides by androgens. Somatostatin (Gross, 1980) and somatostatin mRNA (Argente *et al.*, 1990; Werner *et al.*, 1988) in hypothalamic neurons are increased by testosterone providing one mechanism through which androgens may control growth hormone secretory patterns. Vasopressin (AVP) immunoreactivity (DeVries *et al.*, 1985; Miller *et al.*, 1992), AVP mRNA (Miller *et al.*, 1992), and galanin mRNA (Miller *et al.*, 1993a) are decreased in the bed nucleus of the stria terminalis (BNST) by long-term castration. Recent evidence also suggests that androgens suppress corticotropin-releasing hormone (CRH) immunoreactivity in the hypothalamic paraventricular nucleus (Almeida *et al.*, 1992; Bingaman *et al.*, 1994) thereby influencing the hypothalamo-pituitary-adrenal response to stress. These neuropeptide systems provide an opportunity to examine androgen receptor-mediated regulation of chemically identified neurons.

It is currently unknown whether androgens are capable of directly regulating

forebrain neuropeptide transmitters. In order for this to occur, AR must be expressed within neuropeptide containing cells of the central nervous system, and an androgen response element should be present upstream of the neuropeptide gene. The following experiment tested for the presence of AR immunoreactivity within somatostatin, galanin, AVP, CRH, and oxytocin neurons in the rat forebrain.

Materials and Methods

Six adult male Sprague/Dawley rats (250-275 g) were given a lethal dose of sodium pentobarbital and perfused through the ascending aorta with 4% paraformaldehyde. Brains were removed and post-fixed overnight in paraformaldehyde. 30 μm-thick coronal sections were cut through the diencephalon on a vibratome (Technical Products Intl., St. Louis, MO). Androgen receptor immunoreactivity was visualized using an immunoaffinity purified rabbit polyclonal antibody to rat/human androgen receptor (Prins *et al.*, 1991). This antibody was generated using a synthetic peptide corresponding to amino acids 1-21 of AR and was the generous gift of Dr. Gail S. Prins (Department of Obstetrics & Gynecology, Univ. of Illinois College of Medicine, Chicago, IL). Free-floating sections were incubated with antiserum diluted at 0.5 ug/ml in 0.1 M phosphate buffered saline (PBS) with 0.25% Triton X-100 (Malinkrodt, Paris, KY) and 2% normal donkey serum for 18-24 hours at 4°C. Sections were incubated with biotinylated donkey anti-rabbit gamma-globulin (1:250; Jackson Immunoresearch, West Grove, PA) and then in streptavidin-horseradish peroxidase (Vector, Burlington, CA). Nickel ammonium

sulfate (25 mg/ml) intensified diaminobenzidine (DAB) was used as the chromagen yielding black-stained cell nuclei. Following the immunohistochemical reaction for AR-IR cells, sections were washed in PBS with 0.25% Triton X-100 for 38-42 hours at 4°C.

The following primary antisera were used for the immunohistochemical detection of neuropeptides: anti-corticotropin-releasing hormone (lot #465) provided by the late Dr. T.L. O'Donohue, anti-arginine vasopressin (48 bleed 10) and anti-oxytocin (45 bleed 10) provided by Dr. S.J. Watson (Univ. of Michigan, Ann Arbor), anti-galanin obtained from Peninsula Labs (San Carlos, CA, RAS-7153N, lot # 006807), and anti-somatostatin obtained from Incstar (Stillwater, MN, lot # 853027). After washing the sections stained for AR-IR, these sections were incubated with antiserum diluted at either 1:1000 (galanin and somatostatin), 1:2000 (CRH), or 1:10,000 (AVP and oxytocin) in 0.1M PBS with .25% Triton X-100 and 2% normal donkey serum for 18-24 hours at 4°C. Immunoreactivity was visualized using a biotinylated secondary antibody, streptavidin-horseradish peroxidase, DAB complex as described above except that nickel ammonium sulfate was not present. Sections were mounted on gel-coated slides.

To verify specificity of AR antibody immunostaining, the AR antibody was preincubated with either the peptide used to generate the AR antibody or a distant peptide prior to exposure to the tissue. The peptide used to generate the antibody (peptide 1-21) competed off specific signal. The distant peptide (peptide 462-478) had no effect on AR-IR. As a control for neuropeptide antisera specificity, 1 ml of each

diluted antiserum was preincubated with 50 μg of its respective synthetic neuropeptide, and the sections were processed as usual to determine whether any residual staining remained. Specific immunohistochemical staining was not observed in these controls. However, it is still possible that these antisera may recognize unidentified antigens containing similar amino acid sequences. Thus, the immunoreactivity detected in this study is more accurately described as AR-like and neuropeptide-like immunoreactivity.

Peptide-IR cells were identified by the presence of brown cytoplasmic immunohistological staining. Cells were considered to be AR-IR if a dark-stained nucleus was discernable. Neurons containing AR-IR and peptide-IR (i.e. double-labelled cells) exhibited a dark-stained nucleus surrounded by a brown cytoplasm. AR-, peptide-, and double-labelled cells were counted throughout nuclei in which colocalization was observed. Every fourth section was sampled and used for evaluation of AR-IR in conjunction with the immunoreactivity of a single peptide.

Results

The distribution of AR-IR was similar to the distribution of AR previously described (Sar and Stumpf, 1975; Handa *et al.*, 1987; Wood and Newman, 1993). AR-IR was abundant in the lateral septum, BNST, medial preoptic area, ventromedial and arcuate nuclei of the hypothalamus, CA1 region of the hippocampus, amygdala, and cortex. AR staining was localized predominantly in cell nuclei. The distribution of somatostatin, galanin, AVP, CRH, and oxytocin overlapped that of AR in many

forebrain regions examined.

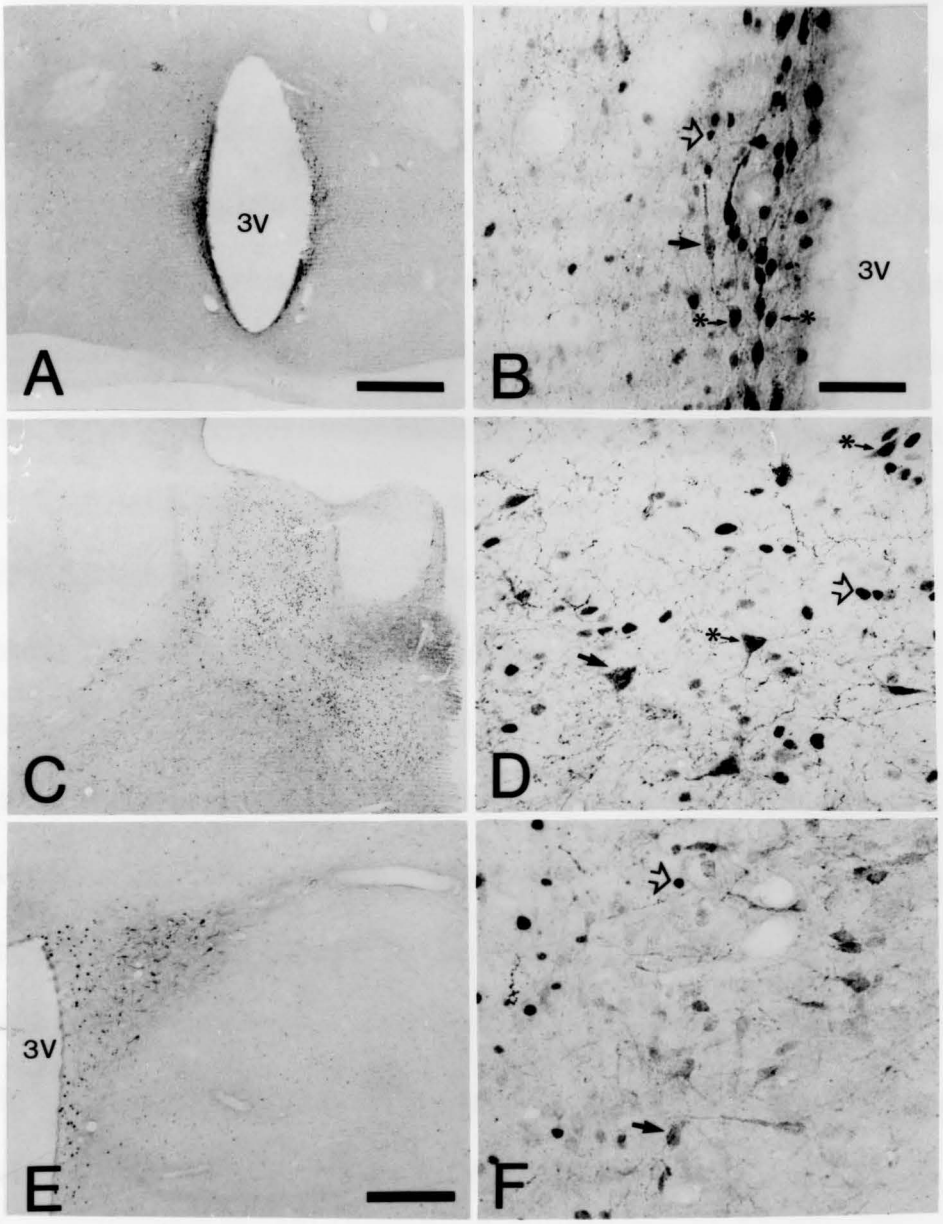
AR was found within somatostatin containing neurons of the periventricular nucleus of the hypothalamus adjacent to the third ventricle (figure 22 A,B). In the periventricular nucleus, double-stained cells represented 78.7$\pm$6.4 % of somatostatin-IR neurons and 54.6$\pm$9.8% of AR-IR neurons (n=3). A few somatostatin containing cells in the paraventricular nucleus of the hypothalamus also expressed AR.

AR-IR was also found within galanin containing cells in the BNST (figure 22 C,D) and the medial and central amygdaloid nuclei. In the BNST double-stained cells represented 39.0$\pm$4.9% of galanin-IR and 1.1$\pm$0.4% of AR-IR neurons (n=3). In the medial amygdala, 44.0 % of galanin expressing cells also expressed AR while 1.25 % of AR-IR cells also contained galanin (n=2). In the central nucleus of the amygdala, double-stained cells represented 23.1% of galanin cells and 1.3% of AR-IR neurons (n=2).

Nuclear staining was not observed in somatostatin- and galanin-IR cells in areas that did not show AR-IR. For example, nuclear staining was not observed in somatostatin-IR neurons of the caudate putamen. Similarly, brown cytoplasmic staining was not seen in areas where AR-IR, but not somatostatin- or galanin-IR cells, are found (e.g. the ventromedial hypothalamus).

AR was not found in CRH (figure 22 E,F), AVP, or oxytocin neurons in the hypothalamus, septum, BNST, or amygdala.

Figure 22. Brightfield photomicrographs showing localization of androgen receptor immunoreactivity within somatostatin containing neurons in the periventricular nucleus of the hypothalamus (A, B), localization of androgen receptor immunoreactivity within galanin expressing neurons in the bed nucleus of the stria terminalis (C, D), and the distribution of androgen receptor immunoreactivity and corticotropin-releasing hormone (CRH) immunoreactivity in the paraventricular nucleus of the hypothalamus (E, F). Androgen receptor immunoreactivity was visualized using nickel intensified diaminobenzidine yielding a black reaction product within cell nuclei (open arrows). Somatostatin (A,B), galanin (C,D), and CRH (E,F) immunoreactivity was visualized as a brown diaminobenzidine reaction product present in the cell cytoplasm (solid arrows). Examples of double-stained cells are indicated with an asterisk (*). 3V=third ventricle. Bar indicates either 500 μm (A,C), 250 μm (E), or 50 μm (B,D,F).


3V
A
3V
B
C
D
3V
E
F

Discussion

These data demonstrate that AR is localized within somatostatin expressing cells of the periventricular nucleus of the hypothalamus. Cells in this region project to the median eminence where somatostatin acts as the major inhibitor of growth hormone secretion from the anterior pituitary (Brazeau *et al.*, 1973). Previous studies have demonstrated a sex difference in the secretory patterns of growth hormone (for rev. see Jansson *et al.*, 1985). Growth hormone secretion is influenced by circulating gonadal steroids such that both stimulatory (growth hormone-releasing hormone, GHRH) and inhibitory (somatostatin) inputs to pituitary growth hormone secretion are modulated by gonadal steroids (Gross, 1980; Werner *et al.*, 1988; Chowen-Breed *et al.*, 1989; Ulloa-aguirre *et al.*, 1990; Zeitler *et al.*, 1990; DeGennaro *et al.*, 1991; Zorilla *et al.*, 1991). Although steroid regulation of growth hormone secretion has yet to be completely defined, it is clear that androgens increase somatostatin mRNA in the periventricular nucleus (Argente *et al.*, 1990; Werner *et al.*, 1988) through an androgen receptor-mediated processes (Argente *et al.*, 1990). Our data suggest that androgens may act within hypothalamic somatostatin expressing neurons to alter somatostatin gene expression.

We have demonstrated that AR is localized with galanin in neurons in the BNST and amygdala. This is consistent with a recent report that androgen regulates galanin gene expression in the BNST (Miller *et al.*, 1993a) and suggests that androgens act within galanin cells of the BNST. Although the function of galanin in the BNST is unknown, the majority of AVP neurons in this region express galanin

mRNA (Miller *et al.*, 1993b). It has been suggested that a potential role for galanin in these neurons may involve the presynaptic modulation of co-released vasopressin (Miller *et al.*, 1993b). Interestingly, we report here that AR-IR was not localized within AVP-IR neurons in any region examined. This contradicts a recent report that AR immunoreactivity is localized within AVP cells of the BNST and MeA (Zhou *et al.*, 1994). However, the Zhou study as well as previous reports of androgen regulation of AVP immunoreactivity (DeVries *et al.*, 1985; Miller *et al.*, 1992) involved the use of colchicine to demonstrate AVP-IR in the BNST and medial amygdala. In this study, we did not use colchicine and thus saw very few AVP-IR cells in the BNST and medial amygdala. The possibility remains that the AR-IR/galanin cells we observed in the BNST and medial amygdala may express AVP but would only display AVP-IR upon prior treatment with colchicine.

Recent studies suggest that androgens play a role in the regulation of the neuroendocrine stress response (Handa *et al.*, 1994). Hypothalamic CRH content and CRH-IR in the paraventricular nucleus are decreased by androgens (Bingaman *et al.*, 1994). However, the absence of AR in the CRH containing cells throughout the paraventricular nucleus suggests that androgens influence CRH neurons via a transsynaptic mechanism. The data also suggest that androgens do not influence the hypothalamo-pituitary-adrenal response to stress through direct actions on oxytocin or AVP cells of the paraventricular nucleus. AR-IR was not found within AVP or oxytocin expressing neurons in any subregion of the parvocellular or magnocellular paraventricular nucleus.

In summary, we have used a dual immunohistochemical technique to determine whether neuropeptide containing cells contain the intracellular machinery to be directly regulated by androgens. Using this technique, AR-IR was found within somatostatin expressing cells of the hypothalamic periventricular nucleus and galanin expressing cells of the BNST and amygdala. Colocalization of AR-IR and AVP, CRH, or oxytocin was not observed in neurons of the rat forebrain. In light of the fact that colchicine was not used in this study, the possibility exists that AR-IR was not observed in AVP, CRH, or oxytocin containing neurons because some cell groups that express these peptides may not have been stained. This double immunohistochemical staining technique is a valuable tool in characterizing AR containing cells. However, the great majority of AR containing cells in many limbic brain areas have yet to be characterized in terms of transmitter phenotype.

CHAPTER VI

DIFFERENTIAL EFFECTS OF CIRCULATING ANDROGEN ON NEUROENDOCRINE AND BEHAVIORAL RESPONSES TO CONDITIONAL STRESS

Summary

The purpose of this study was to examine the effect of circulating androgens on neuroendocrine, autonomic, and behavioral responses to stress. In rats stress produces increases in plasma ACTH, corticosterone, prolactin, and renin, increases in defecation, and characteristic behavioral changes. Androgens may play an important role in the expression of these responses. The effects of conditioned stress were studied in male Sprague/Dawley rats that were gonadectomized with or without dihydrotestosterone (DHT) treatment or received sham surgeries. Animals were stressed three weeks after surgery. The ACTH response to conditioned stress was significantly potentiated ($p < .01$) in gonadectomized males compared to sham-operated and gonadectomized, DHT-treated animals. In stressed rats, plasma corticosterone levels were significantly higher ($p < .05$) in gonadectomized animals compared to DHT-treated castrates. The prolactin response to stress was decreased ($p < .01$) in gonadectomized males compared to sham and gonadectomized, DHT-treated rats. The stress-induced increases in plasma renin activity and concentration

were not altered by gonadectomy or DHT. Non-stressed DHT-treated castrates exhibited more "fear-like" behavior (e.g., more freezing and less sniffing) compared to non-stressed sham and gonadectomized animals. However, conditioned stress produced the same behavioral effects in all treatment groups. The results demonstrate that the ACTH/corticosterone, prolactin, and behavioral responses to a psychological stressor are differentially regulated by circulating androgens.

Introduction

Aversive or anxiogenic stimuli cause biological changes that have been termed the stress response. This so-called stress response is characterized by well documented neuroendocrine, autonomic, and behavioral reactions that serve to preserve the integrity of the animal. Almost all forms of stress produce increases in plasma adrenocorticotropic hormone (ACTH), corticosterone, prolactin, and renin (for rev. see Van de Kar *et al.*, 1991). Enhanced release of corticosterone and ACTH reflects activation of the hypothalamo-pituitary-adrenal axis. Prolactin release is regarded as a reliable index of the stress response, although the physiological significance of prolactin release during stress is unclear. Stress-induced release of renin from the kidney indicates that the renin-angiotensin system is stimulated. The renin-angiotensin system plays an essential role in the regulation of blood pressure and electrolyte balance (Imagana *et al.*, 1984; Churchill *et al.*, 1978). In most species anxiogenic stimuli cause increases in defecation suggesting altered autonomic output to the gastrointestinal system (Miyata *et al.*, 1992). Behavioral indicators of

stress in the rat include an increase in freezing and a decrease in rearing, grooming, sniffing, and locomotor activities (e.g., Rittenhouse *et al.*, 1992; Britton *et al.*, 1982).

A limited number of studies have examined the effects of gonadal steroids on the stress response. A sex difference in basal corticosterone titers and in the corticosterone response to ether stress has been reported (Kitay, 1961). Females have higher basal corticosterone levels and enhanced corticosterone responses to stress compared to males. Several studies suggest that this sex difference arises, in part, as a result of estrogen acting to enhance hypothalamo-pituitary-adrenal activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992). Conversely, circulating androgens appear to inhibit ACTH and corticosterone responses to stress. Gonadectomized male rats exhibit enhanced corticosterone and ACTH responses to foot shock and open field stressors (Handa *et al.*, 1994). Treatment of gonadectomized rats with testosterone or the non-aromatizable androgen, dihydrotestosterone (DHT), normalizes the ACTH and corticosterone responses to these stressors. Furthermore, in male rats the magnitude of the corticosterone response to immobilization is negatively correlated with the levels of circulating testosterone (Viau and Meaney, 1991b).

As noted above, most studies investigating the effect of gonadal hormones on the stress response have sampled only one or two parameters of the stress response. In the following experiment we examined the effects of circulating androgens on neuroendocrine, autonomic, and behavioral indices of stress. It is important to determine whether androgens selectively or globally modulate the expression of

various parameters of the stress response. The effects of conditioned stress were examined in male rats that were gonadectomized or gonadectomized and treated with dihydrotestosterone. We tested whether gonadal androgens alter the corticosterone and ACTH responses to conditioned stress as has been shown to occur in response to footshock and open field stressors (Handa *et al.*, 1994). The effect of androgens on stress-induced increases in plasma prolactin, renin concentration, and renin activity also were examined. In addition, this experiment examined the influence of androgens on the stress-induced changes in defecation and several behavioral parameters associated with fear or anxiety.

Materials and Methods

Animals

Male Sprague/Dawley rats (200-250 g) were purchased from Harlan (Indianapolis, IN). Sprague/Dawley rats were used rather than Fischer 344 (F344) rats due to the great amount of animal handling inherent to the conditioning procedure. It is difficult to achieve very low baseline hormone levels in control animals using the inbred F344 strain. Animals were housed, 2 per cage, in temperature (22-24°C) and humidity (50-55%) controlled rooms. Animals were kept on a 12:12 h light:dark cycle with lights on at 0700h. Food and water were available ad libitum. All experimental protocols were approved by Loyola University Animal Care and Use Committee, and were conducted according to the NIH Guide for the Care and Use of Laboratory Animals.

Surgery

Animals were bilaterally gonadectomized under halothane anesthesia. One half of the gonadectomized animals were administered dihydrotestosterone (DHT) via a 2.5 cm Silastic capsule containing crystalline DHT propionate subcutaneously implanted in each animal's back (GDX + DHT). The remainder of the gonadectomized animals received a sham incision (GDX). Control animals received the same surgeries except that the gonads were not removed and capsules were not implanted in their backs (SHAM).

Conditioned Stress Paradigm

Training and testing were conducted in a rectangular chamber with a grid floor composed of stainless steel rods (7.6 mm in diameter) spaced 12.7 mm apart. The walls and ceilings of the apparatus were made of Plexiglas and illuminated by a 20-watt fluorescent lamp. The chamber was located inside a sound-proof box equipped with a one-way mirror so that the animal's behavior could be videotaped. Scrambled current shock was delivered through the grid floor.

Rats were randomly assigned to treatment groups (control versus stress). Cage mates received the same treatment. Cage mates were tested sequentially and at the same time of day (between 10:00 and 13:30 h) on 4 consecutive days. All rats were handled 2 minutes/day on each of 4 consecutive days prior to training. Training was begun 18 days after surgery. On each of 4 consecutive days, the rats were transported to the conditioning chamber in a transfer cage identical to their home

cage. The rats remained for 5 minutes in the transfer cage prior to placement in the conditioning chamber. For the first 3 days, the experimental (stressed) animals received a 10 sec inescapable foot shock (0.8 mA) that was initiated ten minutes following placement in the chamber. Control rats were treated identically except that they were never shocked. The rats were then returned to their home cage. On the fourth day, animals were placed in the test chamber for 10 minutes but did not receive a foot shock. At the end of the 10 minutes, the rats were removed, transported to an adjacent room and killed by decapitation. Trunk blood was collected for hormone analysis into tubes containing 0.5 ml of 0.3 M EDTA, pH 7.4. Plasma samples were stored at -70°C until assayed for various hormones. On the fourth day, the fecal boli in the test chamber were counted after the removal of each animal. In addition, animals were video-recorded during their final 10-minute interval in the test chamber. Later, videotapes were viewed, and the duration and frequency of freezing, sniffing, grooming, rearing, and locomotor behaviors were scored using a computer program developed by Dr. Andrew D. Levy in the laboratory of Dr. Louis D. Van de Kar (Loyola University, Dept. of Pharmacol. and Exp. Ther.).

Biochemical Determinations

Plasma corticosterone and ACTH assays were performed on unextracted plasma samples according to standard laboratory procedures as previously described in detail (Lorens and Van de Kar, 1987; Carnes *et al.*, 1986). Corticosterone was

assayed using antiserum from ICN Biomedicals, Inc. (Casa Mesa, CA). Data were analyzed by the computer program RIA-AID (Robert Maciel Assoc., Arlington, MA). The sensitivity was 0.01 ng/tube, and the intra-assay variance was 4.5%. For measurement of this and all subsequent hormones all values were obtained in one assay to avoid inter-assay variance. The ACTH antiserum was purchased from IgG Corp. (Nashville, TN). The ACTH antibody recognizes amino acids 5-18. The antibody does not recognize α-MSH, β-endorphin, β-lipotropin, ACTH 1-24 or ACTH 1-16 amide. The ACTH for standards (1-39) was obtained from Calbiochem and ^{125}I-labelled ACTH was obtained from Incstar (Stillwater, MN). The sensitivity of the ACTH assay was 0.25 pg/tube, and the intra-assay variance was 4.2%.

Prolactin radioimmunoassay (RIA) was performed with reagents provided by the NIADDK. Anti-rat prolactin serum S-9 was used at a dilution of 1:25,500 as described previously (Van de Kar and Bethea, 1982). The standards were made from the rat prolactin reference preparation NIADDK-rprl-RP-3. Anti-rabbit IgG (Calbiochem, San Diego, CA) was used to separate the free from the bound tracer. The sensitivity of the assay was 0.02 ng/tube, and the intra-assay variability was 4.8%.

Plasma renin activity was measured using a RIA for generated angiotensin I (ANG I) as described previously (Richardson-Morton *et al.*, 1989). For the assay of *plasma renin concentration*, a saturating concentration of exogenous renin substrate (angiotensinogen) was incubated with plasma renin to generate ANG I at maximal velocity. Plasma renin substrate was obtained from rats that were nephrectomized

and received a dexamethasone injection (0.2 mg/rat) 24 h before sacrifice. The procedure was described previously in detail (Richardson-Morton *et al.*, 1989). RIA of ANG I was conducted with antiserum produced in our laboratory (Richardson-Morton *et al.*, 1989). The dilution of antiserum was 1:16,000. The RIA sensitivity limit was 13.5 pg ANG I/tube. The intra-assay variability was 4.4%.

Plasma testosterone levels were evaluated by RIA using a ^{125}I-testosterone kit (ICN Biomedicals, Inc., Casa Mesa, CA). The standard curve ranged from 5 to 500 pg/tube. Bound and free steroid were separated using a goat anti-rabbit gamma-globulin. The RIA sensitivity limit was 43 pg/tube. The intra-assay coefficient of variance was 5.1%.

For *plasma DHT* determination, steroids were extracted from samples using hexane:ethyl acetate (3:2) and separated on microcelite columns (ICN Biomedicals, Inc.) using an isooctane-ethyl acetate system. DHT was eluted from the column with 5% ethyl acetate in isooctane. Recovery of DHT was 75.9 $\pm$ 1.3%. Plasma DHT was determined by RIA using a ^{3}H-dihydrotestosterone RIA kit (ICN Biomedicals, Inc.). The standard curve ranged from 10 to 500 pg/tube. Bound and free steroid were separated using dextran-coated charcoal. The RIA sensitivity limit was 49 pg/tube. The intra-assay coefficient of variance was 7.5 %.

Statistics

The data are represented as the group means and the standard errors of the mean (S.E.M.). Data were analyzed by two-way analysis of variance across the two

treatments (androgen status x stress/control), and Newman-Keuls' test was used for post hoc analysis (Winer, 1977) using a computer program (NWA STATPAK, Portland, OR). Plasma testosterone and DHT levels were analyzed by a two-tailed t-test (Winer, 1977). A p value of < 0.05 was considered significant.

Results

Plasma testosterone and DHT concentrations are shown in Table 5. Gonadectomy reduced circulating testosterone, and hormone treatment increased DHT titers.

Plasma ACTH concentration was significantly elevated ($p < .01$) in all conditioned stress groups compared to the corresponding non-stressed control groups (Figure 23A). In GDX animals, the plasma ACTH levels following conditioned stress were significantly greater ($p < .01$) than in stressed intact (i.e. sham) animals. The ACTH response to conditioned stress in DHT-treated castrates did not differ from that of sham animals. Corticosterone levels were increased in all conditioned stress groups compared to non-stressed control groups (Figure 23B). The increases were statistically significant ($p < .01$) in the GDX and sham groups. The statistical analysis indicated that no significant surgery x stress interaction occurred ($F_{2,37}=2.05$, $p<0.14$). However, a post hoc Newman Keuls' test revealed that following conditioned stress, plasma corticosterone levels were significantly increased ($p < .05$) in GDX male rats as compared to DHT-treated castrates. There were no significant differences in plasma ACTH and corticosterone among the treatment groups in non-

Table 5. Plasma testosterone and dihydrotestosterone (DHT) levels in sham, gonadectomized, and gonadectomized, DHT-treated male Sprague/Dawley rats determined by radioimmunoassay.[a] Animals were either conditioned stressed or were non-stressed controls.

Treatment[b]	Testosterone (n)[c,d]	DHT (n)[c,d]
SHAM/CONTROL	1.0 ± 0.2 (6)	0.36 ± .04 (8)
SHAM/STRESS	2.4 ± 0.3 (7)[e*]	0.49 ± .05 (8)
GDX/CONTROL	ND (8)	0.45 ± .05 (8)
GDX/STRESS	ND (8)	0.43 ± .03 (7)
GDX + DHT/CONTROL	ND (8)	0.95 ± .07 (8)[f#]
GDX + DHT/STRESS	ND (8)	0.97 ± .06 (8)[f#]

[a]Animals were gonadectomized 3 weeks prior to sacrifice. DHT was administered at the time of gonadectomy using a 2.5 cm Silastic capsule containing crystalline DHT propionate implanted under the skin of each animal's back.

[b]SHAM, sham-operated; GDX, gonadectomized; GDX + DHT, gonadectomized, DHT-treated; CONTROL, non-stressed control; STRESS, conditioned stressed

[c]values expressed in ng/ml

[d]n, number of animals per group

[e]Plasma testosterone levels of SHAM/STRESS animals may be significantly higher than those of SHAM/CONTROL rats due to cross-reactivity of the testosterone antibody with corticosterone.

[f]The measured levels of plasma DHT may reflect cross-reactivity of the DHT antibody with corticosterone. Also, the levels of plasma DHT measured in SHAM and GDX animals were very near the limit of detectability and therefore may not be reliable.

ND designates values too low to detect. The assay limits of detectability were 0.86 ng/ml for testosterone and 0.22 ng/ml for DHT.

* Plasma testosterone levels of SHAM/STRESS animals were significantly ($p < .01$) different from SHAM/CONTROL rats.

Plasma DHT levels of GDX + DHT/CONTROL and GDX + DHT/STRESS animals were significantly ($p < .01$) different from SHAM/CONTROL and SHAM/STRESS animals.

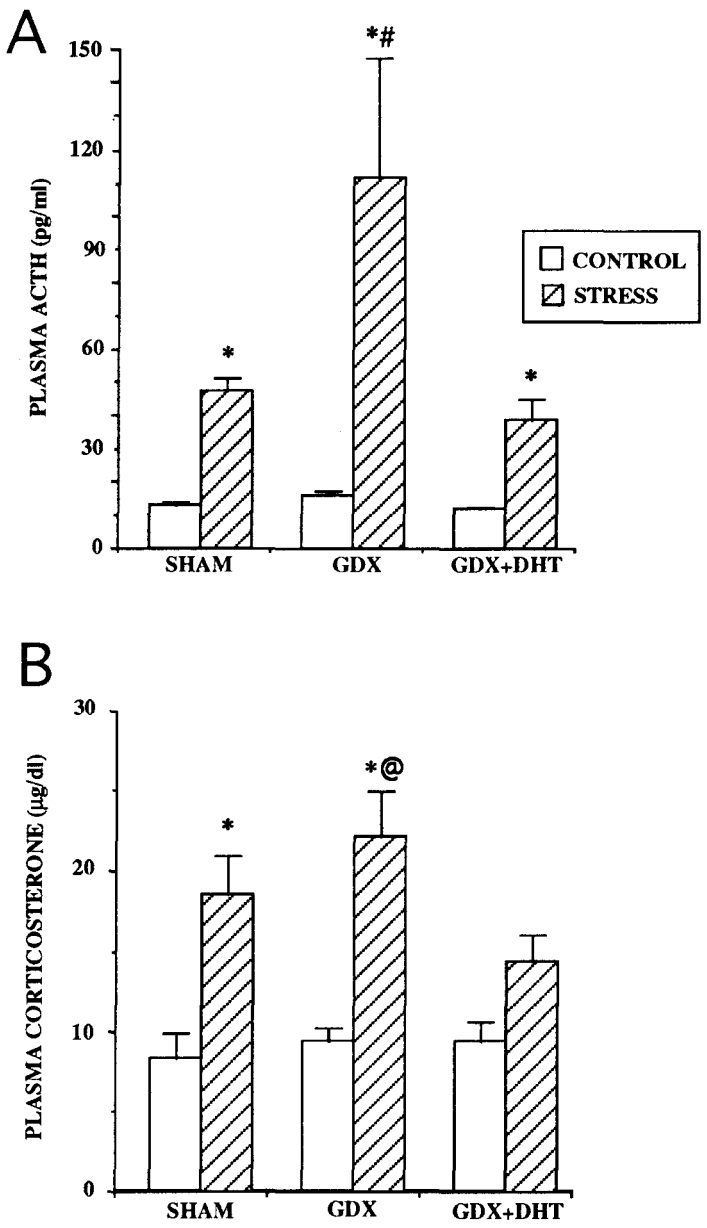


Figure 23. Effect of gonadectomy and androgen replacement on plasma ACTH (A) and corticosterone (B) concentration in conditioned stress and non-stressed control rats. Animals were either sham gonadectomized (SHAM), gonadectomized (GDX), or gonadectomized, dihydrotestosterone-treated (GDX + DHT). The data represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7-8 rats per group. * designates groups which are significantly different from the corresponding non-stressed control group ($p < .01$). # designates groups which are significantly different from sham stress and GDX+DHT stress groups ($p < .01$). @ designates groups which are significantly different from the GDX+DHT stress (but not the sham stress) group ($p < .05$).

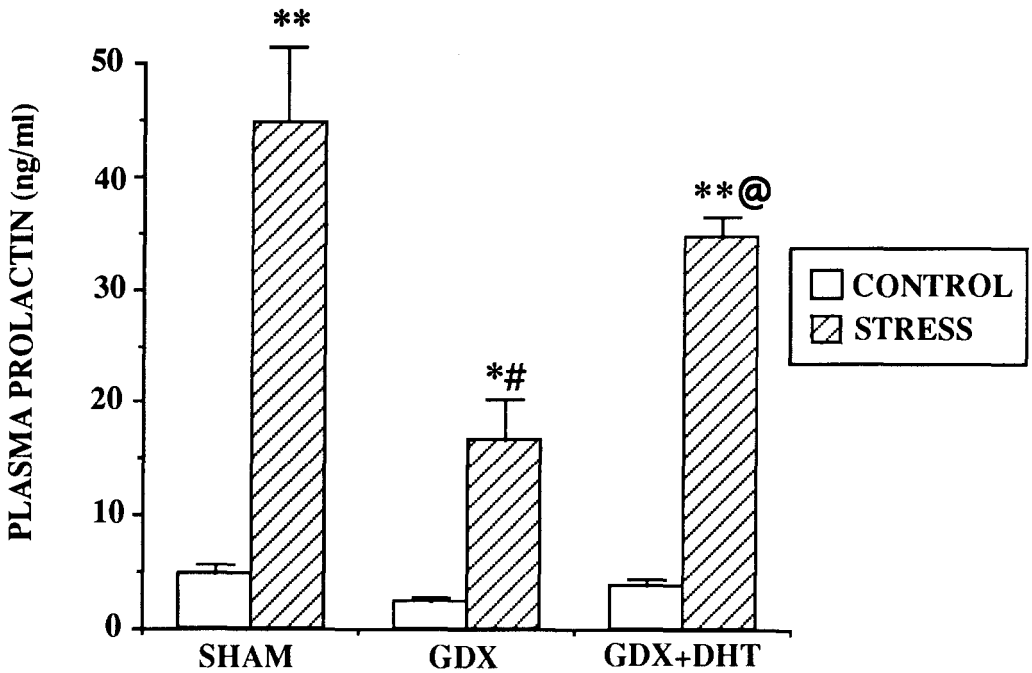


Figure 24. Effect of gonadectomy and androgen replacement on plasma prolactin concentration in conditioned stress and non-stressed control rats. Animals were either sham gonadectomized (SHAM), gonadectomized (GDX), or gonadectomized, dihydrotestosterone-treated (GDX+DHT). The data represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7-8 rats per group. ** designates groups which are significantly different from the corresponding non-stressed control group ($p < .01$). * designates groups which are significantly different from the corresponding non-stressed control group ($p < .05$). # designates groups which are significantly different from sham stress and GDX+DHT stress groups ($p < .01$). @ designates groups which are significantly different from sham stress group ($p < .05$).

stressed control animals.

Plasma prolactin concentrations were significantly increased ($p < .01$) in all stress groups compared to non-stressed control groups (Figure 24). The magnitude of the stress-induced increase in plasma prolactin concentration was significantly lower ($p < .01$) in castrates than in sham-operated animals and DHT-treated castrates. The plasma prolactin concentration following conditioned stress was significantly lower ($p < .05$) in DHT-treated castrates compared to sham-treated animals.

Plasma renin activity and plasma renin concentration were significantly increased ($p < .05$) in all conditioned stress groups compared to the corresponding non-stressed control groups (Figure 25). Plasma renin activity and concentration were not significantly different among the treatment groups in either stressed or unstressed animals.

The number of fecal boli defecated in the chamber during the 10 minute testing procedure was increased in all conditioned stress groups compared to non-stressed control groups (Figure 26). The increases were statistically significant ($p < .01$) in the GDX and GDX+DHT groups. Conditioned stress GDX+DHT rats defecated a significantly greater ($p < .05$) number of boli in the chamber compared to stressed sham animals. No significant differences were seen between castrates and DHT-treated castrates in either stressed rats or in non-stressed controls.

The duration of freezing behavior was significantly increased ($p < 0.01$) in all conditioned stressed groups compared to non-stressed control groups (Figure 27). Sniffing duration, grooming duration, rearing duration and frequency, and locomotor

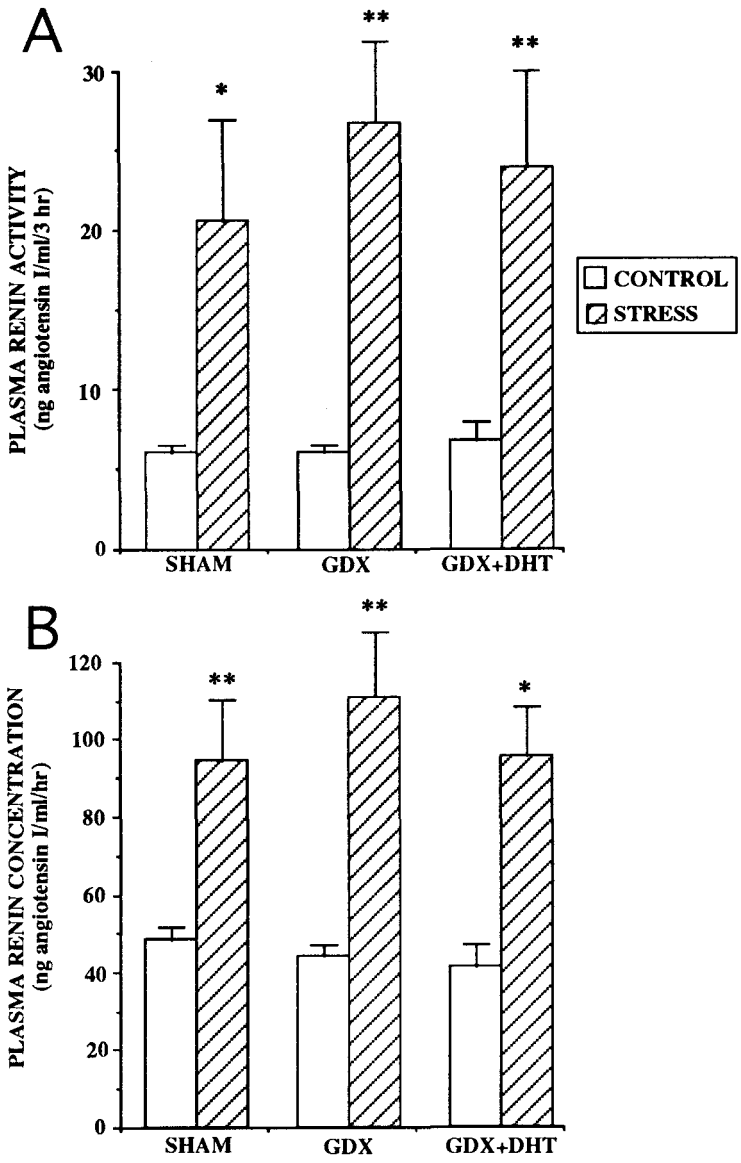


Figure 25. Effect of gonadectomy and androgen replacement on plasma renin activity (A) and plasma renin concentration (B) in conditioned stress and non-stressed control rats. Animals were either sham gonadectomized (SHAM), gonadectomized (GDX), or gonadectomized, dihydrotestosterone-treated (GDX+DHT). The data represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7-8 rats per group. ** designates groups which are significantly different from the corresponding non-stressed control group ($p < .01$). * designates groups which are significantly different from the corresponding non-stressed controls ($p < .05$).

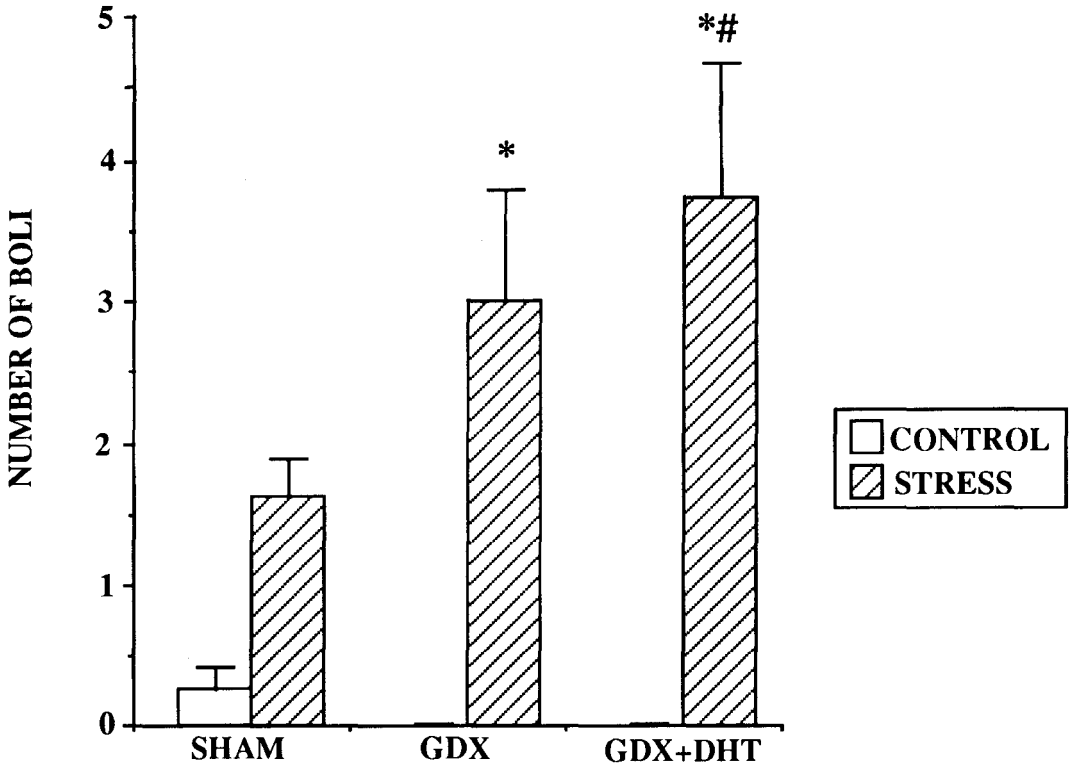


Figure 26. Effect of gonadectomy and androgen replacement on defecation in conditioned stress and non-stressed control rats. Animals were either sham gonadectomized (SHAM), gonadectomized (GDX), or gonadectomized, dihydrotestosterone-treated (GDX+DHT). The data represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7-8 rats per group. * designates groups which are significantly different from the corresponding non-stressed control groups ($p < .01$). # designates groups which are significantly different from the sham stress group ($p < .05$).

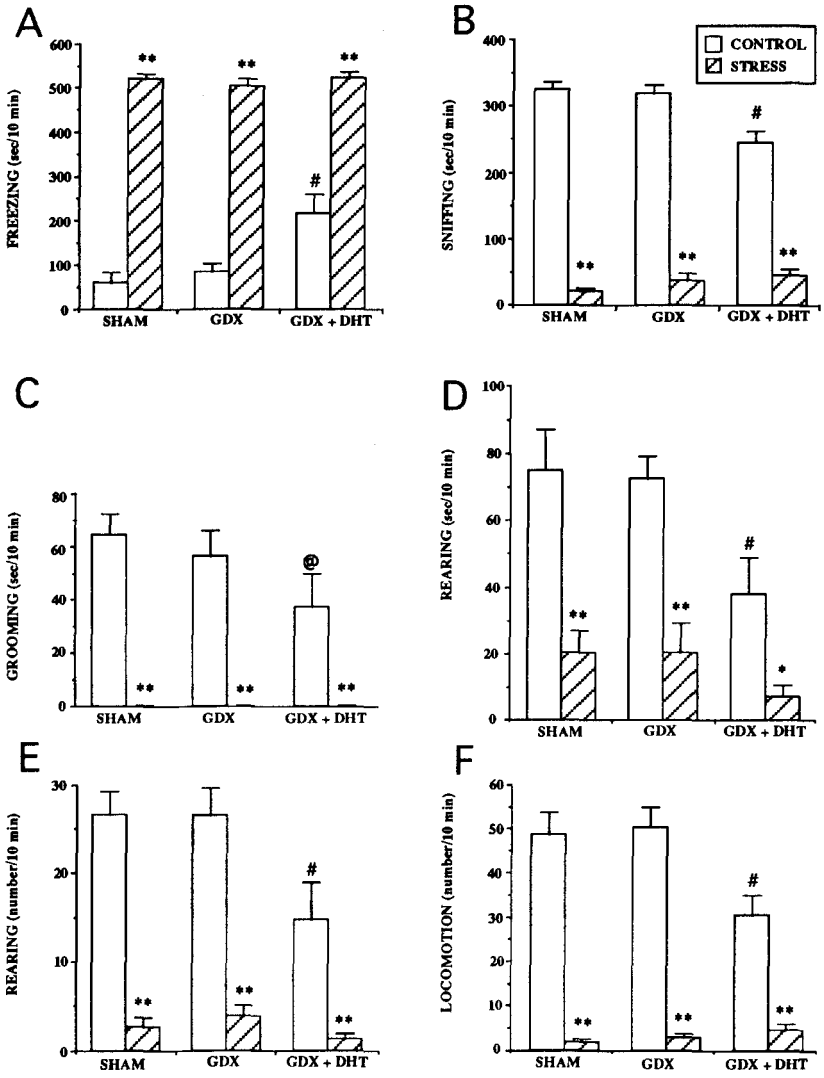


Figure 27. Effect of gonadectomy and androgen replacement on behavioral responses to conditioned stress. The duration (A,B,C,D) and frequency (E,F) of freezing (A), sniffing (B), grooming (C), rearing (D,E), and locomotion (F) were scored from videotapes of conditioned stress and non-stressed control rats over a period of 10 minutes in the testing chamber. Animals were either sham gonadectomized (SHAM), gonadectomized (GDX), or gonadectomized, dihydrotestosterone-treated (GDX+DHT). The data represent the mean $\pm$ the standard error of the mean (S.E.M.) of 7-8 rats per group. ** designates groups which are significantly different from the corresponding non-stressed control ($p<0.01$). * designates groups which are significantly different from the corresponding non-stressed control ($p<0.05$). # designates groups which are significantly different from sham and GDX non-stressed controls ($p<0.01$). @ designates groups which are significantly different from sham non-stressed controls ($p<0.05$).

frequency were significantly decreased ($p<0.01$) in all conditioned stress groups compared to non-stressed controls. No differences were seen among the treatment groups in animals exposed to conditioned stress. Non-stressed DHT-treated castratesexhibited more freezing ($p<0.01$) and less sniffing ($p<0.01$), grooming ($p<0.05$), rearing ($p<0.01$), and locomotor ($p<0.01$) activities compared to non-stressed sham and GDX animals.

Discussion

These results indicate that the ACTH and corticosterone responses to conditioned stress are increased in castrated male rats. Furthermore, androgen treatment normalizes these responses to conditioned stress. This suggests that androgens tonically suppress ACTH and corticosterone responses to stress. The data are consistent with previous studies which demonstrated that gonadectomy increases the ACTH (Coyne and Kitay, 1971; Handa *et al.*, 1994) and corticosterone (Handa *et al.*, 1994) responses of male rats to ether (Coyne and Kitay, 1971), footshock (Handa *et al.*, 1994) and open field (Handa *et al.*, 1994) stressors. The hypothesis that androgens inhibit hypothalamo-pituitary-adrenal responses to stress is further supported by data showing that the magnitude of the corticosterone response to immobilization is negatively correlated with plasma testosterone titers (Viau and Meaney, 1991b). The post-stress plasma corticosterone levels in gonadectomized male rats were significantly increased compared to DHT-treated castrates. Following stress, plasma corticosterone levels in gonadectomized male rats were increased

compared to sham-operated animals, but the effect was not statistically significant. The level of plasma DHT in the DHT-treated castrates was significantly higher than that of sham-operated male rats (Table 5). Thus, the androgen effect on post-stress corticosterone levels could be more apparent in the DHT-treated castrates than in sham-operated animals because of the dampening effect of DHT. It is also possible that corticosterone levels are still rising and a maximum treatment effect has not yet been reached.

Several possible mechanisms could explain the enhanced corticosterone and ACTH responses to stress in the gonadectomized male. Androgens could alter the negative feedback of the hypothalamo-pituitary-adrenal axis. However, castrated male rats exhibit no changes in either concentration or affinity of glucocorticoid or mineralocorticoid receptors in the hippocampus or hypothalamus (Handa *et al.*, 1994). Also, anterior pituitary sensitivity to CRH is not altered in gonadectomized male rats (Handa *et al.*, 1994). However, hypothalamic CRH content and CRH immunoreactivity in the paraventricular nucleus of the hypothalamus are increased in gonadectomized male rats (Bingaman *et al.*, 1994). Dihydrotestosterone treatment of castrates normalizes hypothalamic CRH. Androgens probably influence hypothalamic CRH activity through a transsynaptic mechanism. This is suggested by data showing that androgen receptor immunoreactivity is not located within CRH neurons of the paraventricular nucleus (Bingaman *et al.*, in press). However, androgen receptors are abundant in adjacent regions of the hypothalamus, the limbic system, and the brainstem (Handa *et al.*, 1987). Many of these regions are known to provide direct

input to the paraventricular nucleus (for rev. see Swanson and Sawchenko, 1983).

The effect of gonadectomy on the ACTH and corticosterone responses to conditioned stress were quite robust. This is important in light of the fact that the conditioning paradigm employed in this study involved exposure of the animal to stress on 4 consecutive days. Previous studies used only an acute single exposure to a stressor (Coyne and Kitay, 1971; Viau and Meaney, 1991b; Handa *et al.*, 1994). Thus, the robust effect of androgens observed on the fourth day of exposure to the stressor suggests that the effect is functionally important.

The data indicate that plasma prolactin is dramatically increased in response to conditioned stress. The rise in plasma prolactin that occurs in response to conditioned stress is significantly attenuated in castrated male rats. This is not completely surprising since gonadectomized male rats have decreased basal prolactin titers compared to intact animals (Shin *et al.*, 1974; McNeilly *et al.*, 1980; Brar *et al.*, 1985). Furthermore, administration of testosterone to gonadectomized rats leads to an increase in basal circulating prolactin (Shin *et al.*, 1974; Brar *et al.*, 1985; Kalra *et al.*, 1973; Weidenbach *et al.*, 1980). In the current study, a small decrease in plasma prolactin was observed in control, gonadectomized rats. This decrease was similar in magnitude to that reported previously (Brar *et al.*, 1985). Two-way anova showed a significant effect of androgens on plasma prolactin. However, post-hoc Newman-Keuls' analysis did not detect the small change in basal plasma prolactin as statistically significant due to the large changes observed in plasma prolactin following stress.

Pituitary prolactin secretion is under the control of both prolactin-releasing factors and a prolactin-inhibiting factor. Many putative prolactin-releasing factors have been identified, e.g. vasoactive intestinal polypeptide, thyrotropin-releasing hormone, oxytocin, and angiotensin II (for review see Ben-Jonathon *et al.*, 1989). The major candidate for a prolactin-inhibiting factor is dopamine (Ben-Jonathon, 1985). Gonadectomized male rats have an altered episodic pattern of prolactin secretion (Grosser and Robaire, 1988) and a decreased pituitary prolactin content (Herbert *et al.*, 1977). The pulsatile nature of prolactin secretion is thought to be controlled by central mechanisms since the pulsatile plasma prolactin patterns are no longer found following sectioning of the pituitary stalk (Wehrenberg and Ferin, 1982). This suggests that circulating androgens influence the central mechanisms controlling pituitary prolactin secretion. However, a pituitary site of androgen action is still possible.

Another mechanism by which androgens could affect pituitary prolactin secretion is through the aromatization of testosterone to estrogen. Castrated rats treated with DHT exhibited higher stress-induced prolactin levels compared to gonadectomized males. However, administration of DHT was not sufficient to restore post-stress prolactin titers to that of stressed sham animals even though plasma DHT levels were higher than in intact (i.e., sham-operated) animals. Intact animals have circulating testosterone while DHT-treated castrates do not. Testosterone can either bind to the androgen receptor, be reduced to DHT and bind to the androgen receptor, or can be aromatized to 17β-estradiol and bind to the estrogen receptor (for review

see McEwen and Parsons, 1982). Since DHT cannot be aromatized to 17β-estradiol, this steroid acts only through the androgen receptor. Thus, our data are consistent with the hypothesis that testosterone increases prolactin release, in part, through aromatization to 17β-estradiol. Estrogen treatment of male or female rats results in anterior pituitary gland growth and hyperprolactinemia (Chen and Meites, 1970; Ajika *et al.*, 1972; De Lean *et al.*, 1977; Elias and Weiner, 1987; Cronin *et al.*, 1982; Perez *et al.*, 1986; Handa and Rodriguez, 1991). This is, in part, a result of lactotroph hypertrophy and hyperplasia as a consequence of decreased hypothalamic dopamine secretion (Elias and Weiner, 1987; Cronin *et al.*, 1982; Perez *et al.*, 1986). There is evidence demonstrating a direct anterior pituitary site of action of estrogen on prolactin secretion (Palka *et al.*, 1966; Nicoll and Meites, 1964; Raymond *et al.*, 1978). However, the possibility that the hypothalamus is also an important site of control of prolactin secretion by estrogens cannot be excluded (Kanematsu and Sawyer, 1963; Ramirez and McCann, 1964; Ahren *et al.*, 1971; Hokfelt and Fuxe, 1972). It also remains to be determined whether androgens and estrogens act synergistically to influence anterior pituitary physiology.

It is of interest to speculate on the physiological significance of androgen's effect on post-stress plasma prolactin levels. High levels of plasma prolactin may enhance immune function (Spangelo *et al.*, 1987; Cross *et al.*, 1989; Dardenne *et al.*, 1989; Mukherjee *et al.*, 1990). Thus, the increased prolactin levels observed following stress may protect the organism from stress-induced immune suppression. Following the exposure to stressful stimuli, circulating androgens maintain the

secretion of prolactin and thereby, may indirectly influence the immune system. Moreover, since corticosterone is immunosuppressive (for rev. see Cupps and Fauci, 1982), the increased plasma corticosterone response to stress in the castrate could conspire with a decreased prolactin response to suppress immune function.

Plasma renin activity and plasma renin concentration were significantly increased following conditioned stress in all treatment groups. This is consistent with previous studies (Richardson-Morton *et al.*, 1989, Van de Kar *et al.*, 1984; Van de Kar *et al.*, 1985). Plasma renin activity and concentration were not altered by gonadectomy in either stressed or control castrated animals. The secretion of renin from the kidney following exposure to stressful stimuli is thought to be caused by both sympathetic activation (for rev. see Reid *et al.*, 1986) and a blood-borne renin-releasing factor (Urban *et al.*, 1992). The secretion of this hypothalamic factor is thought to involve both serotonergic (acting via a 5-HT_2 receptor) and catecholaminergic inputs to the PVN (Van de Kar, 1991, Richardson-Morton *et al.*, 1990). Our results suggest that these systems are not altered by circulating androgens.

Conditioned stress led to an increase in the number of fecal boli defecated in the chamber during the 10 minute testing procedure. Circulating androgens do not influence the defecation of stressed rats since no difference was observed between gonadectomized males and DHT-treated castrates. Thus, the autonomic output influencing gastrointestinal activity was not affected by altering circulating androgens.

As expected, conditioned stressed animals displayed a significant increase in

the duration of freezing behavior and significant decreases in the duration of sniffing, grooming, and rearing and the frequency of rearing and locomotor behaviors compared to non-shocked controls. No differences were seen among the treatment groups in animals exposed to conditioned stress. This is not altogether surprising when one considers that the stressed animals display a maximum of stress-like behaviors leaving little room for variation. For example, stressed rats exhibited freezing for approximately 90% of the time they were within the chamber (Figure 27A). Treatment differences were, however, seen among the non-stressed controls. Non-stressed DHT-treated castrates exhibited more freezing and less sniffing, grooming, rearing, and locomotor activities compared to non-shocked intact and gonadectomized animals. Since the control rats were placed in the chamber for four consecutive days (without being shocked), they were exposed to a mild amount of stress inherent to the procedure. This mild stress is evident in plasma corticosterone levels when one compares home cage control rats with rats subjected to this procedure (home cage controls, 0.8 ± 0.2 μg/dL; non-shocked controls, 3.0 ± 0.8 μg/Dl; conditioned stress, $12.9 \pm$ μg/Dl; Van de Kar, unpublished data). Thus, DHT-treated castrates exhibited more "fear-like" behavior in response to this mild stressor compared to intact and castrated rats. Differences between DHT-treated castrates and intact animals could be attributed to the higher levels of DHT in the DHT-treated castrates (Table 5) and/or to the nonaromatizable nature of DHT.

In summary, the data suggest that circulating androgens are necessary for a normal response to conditioned stress. Removal of circulating gonadal androgens

enhances the ACTH and corticosterone responses and attenuates the plasma prolactin response to conditioned stress. Also, androgens may potentiate the behavioral response to stress. The absence of gonadal androgens does not influence plasma renin activity and concentration or defecation following conditioned stress. Thus, circulating androgens differentially modulate the neural systems responsible for expression of various components of the stress response. One major implication of this study shoud be emphasized. To date, most studies investigating the effect of gonadal hormones on the stress response have sampled only one or two parameters of the stress response. This study indicates that circulating andrognes differentially influence various components of the stress response. Examination of only one aspect of the stress response, such as ACTH and/or corticosterone, may be misleading. Depending on the parameter measured, subjects may appear less stressed, more stressed, or unaffected by a particular treatment. Thus, evaluating other neuroendocrine as well as autonomic and behavioral reactions to experimental challenge may be necessary to more accurately determine how an organism is reacting to stress.

CHAPTER VII

GENERAL DISCUSSION

Summary

Circulating gonadal steroids have been demonstrated to influence the hypothalamo-pituitary-adrenal (HPA) axis (Kitay, 1963; Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Coyne and Kitay, 1971; Viau and Meaney, 1991a; Viau and Meaney, 1991b; Burgess and Handa, 1992; Handa *et al.*, 1994). While estrogen enhances HPA activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992), circulating androgens may suppress activity of the HPA axis (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994). The first experiment of this dissertation tested the hypothesis that androgens influence the HPA axis by reducing hypothalamic levels of CRH. It was observed that hypothalamic CRH content is increased in the male rat 3 weeks after bilateral gonadectomy. Treatment of gonadectomized male rats with the non-aromatizable androgen, dihydrotestosterone (DHT), normalizes CRH content to that seen in intact animals. Greater than 10 days of castration was necessary in order to observe a change in hypothalamic CRH content. Furthermore, three weeks after surgery, gonadectomized male rats had significantly more CRH-immunoreactive (IR) cell numbers in the paraventricular nucleus of the hypothalamus (PVN) compared to DHT-treated castrates. Thus, these

data indicate that the effect of androgens on the HPA axis occurs, at least in part, at the level of the hypothalamus.

The second experiment was performed to determine whether the influence of androgens on CRH content and CRH immunoreactivity in the hypothalamus are a consequenc of changes in CRH gene expression. Corticotropin-releasing hormone mRNA levels in the PVN of intact, gonadectomized, and gonadectomized, DHT-treated male rats were evaluated using *in situ* hybridization histochemistry. Corticotropin-releasing hormone mRNA levels in the hypothalamic paraventricular nucleus, however, were not altered 10 days or 3 weeks after gonadectomy. Thus, changes in CRH peptide levels cannot be explained by concurrent changes in mRNA levels or by a transient elevation in CRH mRNA preceding the alteration of peptide content.

The third experiment tested the hypothesis that androgens regulate CRH by acting directly within CRH expressing neurons of the PVN. The regulation of cellular processes by androgens requires the presence of the androgen receptor (AR) which acts as a ligand-responsive transcription factor. In order for androgens to directly regulate hypothalamic CRH, AR must be expressed within CRH containing neurons. Thus, this study tested for the presence of AR immunoreactivity within CRH neurons of the PVN in male rats. The results of this experiment suggest that CRH containing neurons of the PVN do not contain AR immunoreactivity. Thus, it is likely that androgens influence CRH content via a transsynaptic mechanism.

The final study was designed to determine whether circulating androgens

influence neuroendocrine, autonomic, and behavioral responses to a psychological stressor. The effects of conditioned stress were examined in male rats that were intact, gonadectomized, or gonadectomized and treated with DHT. Consistent with previous studies (Coyne and Kitay, 1971; Handa *et al.*, 1994), the ACTH and corticosterone responses to conditioned stress were enhanced in gonadectomized male rats. However, the prolactin response to conditioned stress was suppressed in rats deprived of circulating gonadal androgens. Furthermore, DHT-treatment increased the expression of stress-associated behaviors. Thus, circulating androgens *differentially* influence neuroendocrine and behavioral responses to conditioned stress. Circulating androgens may selectively modulate the neuronal circuitry responsible for mediating various aspects of the stress response.

Possible Mechanisms of Androgen Action

The HPA Axis

Androgens suppress HPA activity via central mechanisms. The loss of circulating gonadal androgens induces an increase in hypothalamic CRH content. This increase may explain the enhanced ACTH and corticosterone responses to physical and psychological stressors previously observed in the gonadectomized male rat (Coyne and Kitay, 1971; Handa *et al.*, 1994). Also, anterior pituitary sensitivity to CRH is not altered in the gonadectomized male rat (Handa *et al.*, 1994). Thus, androgens may act on a central nervous system (CNS) site(s) to alter HPA activity.

Several mechanisms could explain the regulation of hypothalamic CRH by

androgens. Possibilities include an increased synthesis or half-life of CRH or a decrease in axoplasmic transport and/or secretion following gonadectomy. The possibility that axoplasmic transport and secretion of CRH are decreased following castration can be excluded due to the fact that the ACTH and corticosterone responses to stress are increased in the gonadectomized male rat (Coyne and Kitay, 1971; Handa *et al.*, 1994). The possibility remains that synthesis in the soma or degradation of the peptide can be influenced by circulating androgens. Circulating androgens could decrease CRH peptide half-life or reduce the translational efficiency of CRH in parvocellular PVN neurons.

In the second experiment, CRH mRNA levels in the PVN were not altered 10 days or 3 weeks after gonadectomy. However, it is possible that changes in hypothalamic CRH content observed after gonadectomy are the result of an alteration of CRH gene expression. This hypothetical change in CRH gene expression might occur only in a small subset of cells and thus would be masked by a lack of change elsewhere in the PVN. Also, 10 days and 3 weeks after gonadectomy may not be the appropriate time to see such a change.

If the increase in CRH content after gonadectomy is the result of changes in gene expression, one might observe this change by measuring CRH mRNA after a stressor. During homeostasis, CRH is stored in median eminence terminals and is secreted in relatively small amounts in response to a circadian pacemaker (Dallman *et al.*, 1977). The presence of a real or perceived threat to the organism causes secretion of CRH into the hypophysial portal circulation. Following stress, CRH

mRNA increases in the medial parvocellular region of the PVN (Lightman and Young, 1988; Lightman and Young, 1989). This most likely represents an increase in the synthesis of CRH to replenish CRH stores released during stress. Presumably, CRH is synthesized in PVN neurons until the releasable pools of CRH are restored to pre-stress levels. In the castrated male rat, the size of the releasable pools of median eminence CRH may be greater than in the intact male rat. In the final study of this dissertation, the CORT and ACTH responses to stress were enhanced in the castrated male on the fourth day of exposure to a stressor. Thus, it appears that the larger pool of CRH in the castrated male is rapidly regenerated after a stressor. Therefore, if changes in CRH gene expression *are* responsible for androgen's effect on hypothalamic content of this peptide, this may be best observed after a stressor.

Androgen regulation of CRH neurons of the PVN is indirect. Androgen receptor immunoreactivity was not observed within CRH expressing neurons of the PVN. Circulating androgens may act on other CNS neurons to modify levels of glucocorticoid or mineralocorticoid receptors and/or some neuromodulator which ultimately alters the pools of CRH stored in median eminence terminals. The PVN is a highly integrated and regulated cell group (for rev. see Swanson and Sawchenko, 1983), and there are many possible target sites at which steroids could act to ultimately regulate CRH cells within the PVN. Several CNS sites have androgen responsive neurons and project to the PVN. These sites include limbic regions such as the bed nucleus of the stria terminalis (BNST) and the septum as well as brainstem sites such as the midbrain raphe (Sar and Stumpf, 1977; Swanson, 1987).

It is also possible that androgen responsive cells such as those in the hippocampus modify hypothalamic CRH content via a multisynaptic pathway. Interestingly, it has been reported that implantation of the synthetic glucocorticoid, dexamethasone, into the hippocampus results in a slight decrease in the adrenalectomy-induced effects on CRH immunoreactivity (Kovacs *et al.*, 1986) while no effect was seen on CRH mRNA (Kovacs and Mezey, 1987). Androgens may act on the hippocampus to similarly influence hypothalamic CRH.

This putative effect of androgens on the hippocampus may contribute to the enhanced activity of the HPA axis observed in the aged male rat. Extensive research has demonstrated an effect of adrenal steroids on the destruction of hippocampal neurons. ACTH or cortisone treatment of guinea pigs causes necrosis of pyramidal neurons of the hippocampus (Aus der Muhlen and Ockenfels, 1969). Furthermore, in the rat, aging results in the loss of some pyramidal neurons in the hippocampus (Landfield, 1987). This loss can be reduced by adrenalectomy in mid-life (Landfield, 1987). Daily injections of corticosterone to young adult male rats mimicks the neuronal loss observed during aging (Sapolsky *et al.*, 1985). Excitatory amino acids play an important role in this pyramidal cell loss (for rev. see Sapolsky, 1990). Corticosterone exacerbates kainic acid-induced hippocampal damage (Sapolsky, 1986), and glucocorticoids enhance the excitatory amino acid-induced loss of hippocampal neurons in culture (Sapolsky *et al.*, 1988). Moreover, extensive research has provided evidence that the activity of the HPA axis is enhanced in the aging male rat (for rev. see McEwen, 1992). Since there is significant evidence implicating the

hippocampus in the feedback inhibition of the HPA axis (for rev. see Jacobsen and Sapolsky, 1991), the "glucocorticoid cascade hypothesis" of stress and aging was generated in an attempt to formulate a causal relationship between the activity of the HPA axis and the deleterious actions of glucocorticoids on the viability of neurons in the hippocampus (for rev. see Sapolsky et al., 1986). It is possible that circulating androgens contribute to the enhanced activity of the HPA axis observed in the aged male rat. Studies in this dissertation and elsewhere (Critchlow *et al.*, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994) demonstrate that circulating androgens inhibit HPA activity. Moreover, circulating androgens are known to decline in the aging male rat (Kaler and Neaves, 1981). Interestingly, a recent report suggests that circulating androgens protect hippocampal neurons from the N-methyl-D-aspartic acid-(NMDA-) induced loss of membrane activity (Pouliot *et al.*, 1993). This suggests that circulating androgens may influence glutamatergic responses in hippocampal pyramidal neurons to ultimately inhibit HPA activity. Thus, loss of cirulating androgens may contribute to the enhanced activity of the HPA axis in the aging male rat.

The Stress Response

In the final study of this dissertation (Chapter VI), several indices of the stress response were evaluated in terms of possible androgen regulation. The various components of the stress response are controlled by overlapping but different neuronal pathways. For example, serotonin stimulates the secretion of CRH and putative

prolactin- and renin-releasing factors (for rev. see Van de Kar, 1991). CRH secretion is also stimulated by catecholamines and acetylcholine and inhibited by GABA and opioid peptides (for rev. see Owens and Nemeroff, 1991). Furthermore, prolactin release from the pituitary is tonically inhibited by dopamine (Ben-Jonathon, 1985). Thus, the evaluation of several indices of stress may make it possible to gain insight into where and how androgens may be acting to alter the stress response. The absence of circulating gonadal androgens enhanced the ACTH and corticosterone responses and attenuated the prolactin response to conditioned stress. The non-aromatizable androgen, DHT, increased the expression of stress-like behaviors. The renin response to conditioned stress was not altered by circulating androgens.

In addition to the data from this study, hypotheses can be formed based on the results of a study localizing androgen receptor immunoreactivity to peptidergic neurons of the forebrain (Chapter V). In this experiment, androgen receptor immunoreactivity was observed within galanin expressing cells of the ventrolateral BNST. Testosterone regulation of galanin gene expression in the BNST has been reported (Miller et al., 1993a). Cells in the ventrolateral BNST have been demonstrated to project to the PVN (Sawchenko and Swanson, 1983). Ibotenic acid lesion of the ventrolateral BNST attenuates the stress-induced increases in ACTH, corticosterone, and prolactin without influencing the renin response to stress (Gray *et al.*, 1993). Interestingly, infusion of galanin into the PVN attenuates the stress-induced increase in plasma ACTH (Hooi *et al.*, 1990), and galanin has been shown to have a regulatory role in the secretion of prolactin (Koshiyama *et al.*, 1987;

Nordstrom *et al.*, 1987; Ottlecz *et al.*, 1988).

Galanin may alter an organisms response to stress through its actions on other central neurotransmitter systems. In the central nervous system, galanin is known to influence noradrenergic, serotonergic, and cholinergic systems (for rev. see Bartfai *et al.*, 1993). For example, studies in rat indicate that intracerebroventricular injections of galanin potently stimulate the serotonin metabolism in the hippocampus and median eminence (Sundstrom and Melander, 1988). Moreover, galanin appears to increase the affinity of 5-HT_{1A} receptors for serotonin (Fuxe *et al.*, 1988). Substantial evidence suggests that 5-HT_{1A} receptors are involved in the serotonin-induced secretion of ACTH but not in the serotonin-induced secretion of renin (for rev. see Van de Kar, 1991). Furthermore, 5-HT_{1A} receptors may play a role in the expression of CRH-induced behavior (Lazosky and Britton, 1991), a putative analogue of stress-induced behavior (Koob *et al.*, 1993).

Taken together, these studies suggest the possibility that androgens may alter the response to stressful stimuli through its regulation of galaninergic and ultimately serotonergic systems. Clearly, androgens could be acting on any number of neuroanatomical and neurochemical systems to affect changes in the response to stress. However, future studies evaluating androgen regulation of galaninergic and serotonergic transmitter systems may be useful in determining the mechanism(s) by which androgens alter the physiological and behavioral responses to stress.

Implications and Conclusions

The integrity of the HPA axis is essential for a normal response to threatening stimuli. Abnormal funtion of the HPA axis has been associated with psychological disorders such as depression (Kalin and Dawson, 1986) and panic disorder (Henry, 1990). For example, depression is often brought on by stressful episodes (Paykel, 1979) and has been associated with excessive secretion of HPA hormones (Kalin and Dawson, 1986).

In humans, depression and panic disorder occur much more often in females than in males (Weissman and Klerman, 1977; Katschnig and Amering, 1990). Correspondingly, experimental data in rats reveal a sex difference in the corticosterone response to stress (Kitay, 1961). Several studies have indicated that estrogen acts to enhance HPA activity (Kitay, 1963; Viau and Meaney, 1991a; Burgess and Handa, 1992) while androgens may inhibit activity of the HPA axis (Critchlow and Kitay, 1963; Gaskin and Kitay, 1971; Handa *et al.*, 1994; Bingaman *et al.*, 1994). Thus, while estrogen may play an important role in predisposing a female to such disorders, androgens may protect a male from dysfunction of the stress response.

These phenomena are also important in light of the various conditions involving altered gonadal function as well as behaviors which have been linked to HPA dysfunction. For example, Klinefelter's Syndrome, a genetic disorder present in 1 in 1000 male births (Baker *et al.*, 1976), is marked by unusually low testosterone levels throughout life (Schwartz and Root, 1991). Klinefelter males often exhibit

extreme depression (Nielsen *et al.*, 1988; Schwartz and Root, 1991). Also, in the elderly, gonadal hormones are known to diminish (Kaler and Neaves, 1981; Baker *et al.*, 1976) while the HPA response to stress is exaggerated (Sapolsky *et al.*, 1984b). Moreover, a higher incidence of depression is seen among the elderly (Sorensen and Stromgren, 1961). One might contend that other factors (death of a spouse, low income, reduced mobility, etc.) may be the cause of depression in the elderly. However, it is possible that the stress associated with these factors, and the inappropriate biological response to that stress, could be involved in the onset of depression.

It is important to note that most studies investigating the effect of gonadal hormones on the stress response have sampled only one or two parameters of the stress response. In the final study of this dissertation, the effects of circulating androgens on neuroendocrine, autonomic, and behavioral indices of stress were evaluated in order to determine whether androgens selectively or globally modulate the expression of various parameters of the stress response. Interestingly, removal of circulating gonadal androgens enhanced the ACTH and corticosterone responses and attenuated the plasma prolactin response to conditioned stress. Also, while androgens potentiated the behavioral response to stress, the absence of gonadal androgens did not influence plasma renin activity and concentration or defecation following conditioned stress. Circulating androgens *differentially* modulate the neural systems responsible for expression of various components of the stress response. Thus, examination of only one aspect of the stress response such as ACTH and/or corticosterone may be

misleading. Evaluating other neuroendocrine as well as autonomic and behavioral reactions to stress may be necessary in order to determine how an organism is reacting to environmental perturbation. It may be important for researchers to sample several indices of stress in order to gain a more accurate "profile" of the stress response.

It should also be emphasized that gonadal steroids do not cause a female to be "more stressed" or "less stressed" than a male. Gonadal steroids cause males and females to be stressed differently from each other. That is, males may exhibit more stress-like behaviors and less of an HPA response to stress. Furthermore, other hormonal and autonomic responses to stress may be more or less in males compared to females. Thus, females may be more susceptible to depression, not because they are "more stressed" than males beut rather because they respond to stressful stimuli *differentally* than males.

The studies in this dissertation have demonstrated that circulating gonadal androgens play an important role in the regulation of an organism's response to environmental perturbation. Circulating androgens suppress the HPA axis via central mechanisms. In contrast, androgens have a facilitatory effect on prolactin secretion. These effects are quite pronounced when the animal is challenged with a stressor. In the absence of circulating androgens the machinery with which an organism copes with its environment is weakened, and exposure to either physical or psychological stress brings out that weakness. In this manner, an inadequate gonadal steroid secretion can lead to pathological conditions like depression. Future studies of the

site(s) and mechanism(s) of androgen action will not only provide an understanding of gonadal influences on the physiological adaptation to stress, but may be important in providing an understanding of the biological basis underlying behaviors such as depression.

This dissertation also points out the importance of testosterone as a regulator of evolution. Survival of the organism and survival of the species are of utmost importance. Obviously, testosterone acts to contributes to the survival of a species by directly regulating reproduction. However, circulating testosterone may indirectly enhance both survival of the species and survival of the organism through its regulation of the stress response. Since products of the HPA axis inhibit reproductive function (Plas-Rose and Aron, 1981; Sirinathsinghji *et al.*, 1983; Rivier and Vale, 1984; Rivier and Vale 1985b; Rivier *et al.*, 1986; Armstrong, 1986; Sirinathsinghji *et al.*, 1986; Kamel and Kubajak, 1987), circulating testosterone indirectly enhances reproduction by inhibiting HPA activity. In this manner, circulating androgens indirectly enhance survival of the species. Additionally circulating testosterone potentiates the prolactin response to stress. Since prolactin is thought to enhance immune function (Spangelo *et al.*, 1987; Cross *et al.*, 1989; Dardenne *et al.*, 1989; Mukherjee *et al.*, 1990) circulating testosterone may indirectly enhance the survival of the organism through it's regulation of the prolactin response to stressful stimuli. It seems appropiate that testosterone would play this major role in the evolution process. An organism contributes to the evolution of its species during and prior to its reproductive stage. What better mediator of the evolutionary process than

testosterone, a hormone that is only produced in great amounts during a male's reproductive stage. Clearly, circulating androgens represent a major mediator of evolution.

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VITA

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APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

11/9/94
date

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